DEFICIENCY SCREENING AND ANALYSIS OF SEIZURE SUSCEPTIBILITY ENHANCER GENES IN *DROSOPHILA MELANOGASTER*

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Loss of control of neuronal excitability underlies conditions such as seizure and epilepsy, where there is excessive or synchronized firing in the brain. There is evidence that many epilepsies have a genetic basis that either causes or predisposes individuals to seizures. Many animal models have been developed to study the process of epileptogenesis, or the process by which the brain becomes epileptic. *Drosophila*, in particular, has been increasingly used to study neuronal excitability and seizure susceptibility due to its large repertoire of genetic tools and collection of seizure-sensitive and paralytic behavioral mutants. However, while many of these seizure-sensitive mutants show similar phenotypes, many of the mutations are found in genes that have no clear relationship to each other. In order to find genes that enhance excitability and possibly find pathways that link various seizure mutations, we have performed a deficiency screen to search for genes that are enhancers of seizure susceptibility. Bang-sensitive (BS) mutants display a robust seizure phenotype in response to a mechanical or electrical stimulus and we used the weakly semi-dominant BS mutant, *slamdance* (*sda*) as the sensitized background to screen with a set of deficiency mutants that span over chromosome 3R. Through this screen, we found 10 candidate seizure enhancer genes, 8 of which have not been previously linked to a neuronal excitability phenotype. We further analyzed *pumilio* (*pum*), one of the enhancers

found in this screen. *pum* encodes a translational repressor involved in many processes in *Drosophila* development, from larval body axis patterning to roles in nervous system development in later stages. To study the effect of *pum* on seizure susceptibility, we have used behavioral testing and electrophysiology as tools to determine when and where the loss of Pum function leads to enhanced seizure behavior. We have found that Pum functions only in neurons, especially cholinergic and GABAergic neurons, to regulate neuronal excitability and affect seizure susceptibility. We further determined that Pum expression is required both during the larval and pupal stages for proper regulation of neuronal excitability.

BIOGRAPHICAL SKETCH

Hannah was born in Los Angeles, California and grew up in Monterey Park, California all of her life, except for two years where she lived on the opposite side of the nation in Augusta, Georgia between the ages of seven to nine. Once she returned to Monterey Park, she attended Monterey Highlands Elementary School, which had a surprising number of exciting and adventurous science teachers that encouraged Hannah's curiosity of the natural world with activities that ranged from observing microbes in pond water to hiking around Death Valley to learn about ecological conservation and wildlife. She then attended Mark Keppel High School, where she spent her free time reading, playing the piano, playing tennis, as well as hanging out with friends in the Science Club and running biology and chemistry experiments together for fun.

In her junior year of high school, she attended a summer science program called the Young Engineering and Science Scholars at the California Institute of Technology (Caltech) where she spent a month of intense science exploration with a group of like-minded high school students from which she emerged knowing that science was going to be a part of her future. She liked the summer program so much that she ended up spending her undergraduate years at the same institution, majoring in biology. After some time working in the lab of Paul Sternberg, she realized that research and graduate school was more to her liking and interest than going to medical school.

After finishing her undergraduate degree, Hannah then moved to a much different place, Ithaca, New York, to attend graduate school at Cornell University. In the spring of 2004, she joined the lab of Kathleen Whitlock to study hormone

producing cells in the brains of zebrafish, but after the departure of Kate Whitlock from Cornell, Hannah then joined the lab of David Deitcher in 2006 to study seizure susceptibility in a completely different model organism, *Drosophila melanogaster*, during which she developed a great interest in neurobiology and teaching.

For mom, dad, Peter, 할머니, and 할아버지

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BS	bang-sensitive
DLM	dorsal longitudinal muscle
GABA	gamma amino-butyric acid
GF	giant fiber
NMJ	neuromuscular junction

CHAPTER 1

INTRODUCTION

1.1 A brief overview

The regulation of the information flow in the brain through the neurons and their synapses requires a careful balance of excitability and inhibition. Defects in this control that lead to excessive excitability or lack of inhibition can cause abnormal firing in the brain, sometimes resulting in seizure, or even epilepsy. There are numerous conditions that can lead to seizures, such as head injuries, medical conditions, stroke, and mutations in genes. The genetics behind seizure and epilepsy is of great interest because this helps us learn about how particular genes are important in the proper maintenance of activity in the brain from early development to adulthood. Through studies in model organisms, much has been learned about how seizures and epilepsy can develop.

1.2 Advances in studying the genetic basis of epilepsy

A seizure is an event in which there is either excessive or synchronous activity in the brain. This activity can manifest outwardly in a wide range of behaviors, from a short loss of awareness (absence seizure), to convulsions of the entire body (tonic-clonic seizure). The seizures can be limited to specific parts of the brain in cases of partial seizure, or they can spread through the entire brain, which is known as a generalized seizure. Epilepsy occurs when seizures become recurrent and unprovoked, and this condition affects 1% of the population, making it a prevalent neurological disorder [1], [2].

Seizures can be categorized as symptomatic or idiopathic. Symptomatic seizures are those where the cause of seizure is known, such as from head trauma, illness, drugs, and others. In idiopathic seizures, the cause of the seizure is not apparent, and often these are assumed to have an underlying genetic basis [3].

1.2.1 The search for genes underlying epilepsy

In early searches for genes underlying epilepsy, Mendelian genetics was used to discover mutations that caused epilepsies in families, using techniques such as pedigree analysis and linkage mapping. In many of these cases, the mutated gene turned out to encode defective ion channels or channelopathies, such as *SCN1A*, which encodes a subunit of the voltage gated sodium channel, *KCNQ2*, which encodes a delayed rectifier potassium channel, and *GABRG2*, which encodes a gamma-aminobutyric acid (GABA) receptor subunit (reviewed in [4–6]).

More recently, newer techniques have been used to identify genes that had not previously been known to be involved in epilepsy and do not encode ion channels. One example of this is the gene *STXBP1*, a conserved gene also known as MUNC18 and Sec1, which causes early infantile epileptic encephalopathy. This gene was identified from a single patient using comparative genomic hybridization. Unlike the previously identified channelopathies, this gene product is involved in the release of synaptic vesicles [7]. A genome-wide association study was also performed in order to find candidate epilepsy genes among cases of partial-onset epilepsy, though this did not produce any clear candidate genes [8]. Other recent studies identified potential epilepsy genes by using an oligonucleotide assay to identify copy number variants in cases of idiopathic epilepsy [9]. It is suggested that as the field of epilepsy genetics moves forward, more modern techniques will be applied to discover new candidate genes in a high-throughput manner [6].

There are a variety of ways to affect synaptic transmission, which is at the heart of neuronal excitability. These include regulation of second messengers, transcriptional activation, protein trafficking, phosphorylation, RNA editing, and translational control. Defects in any of these processes can result in a number of different diseases and syndromes affecting the nervous system. Often, if a defect

results in an increase of neuronal excitability or lack of suppression of neuronal excitability, this can result in seizure or epilepsy.

1.2.3 Animal models of epilepsy

The search for genes underlying epilepsy is often limited by needing large numbers of people affected by the same type of epilepsy and having to work with epilepsies already in existence. Animal models offer many other methods of searching for novel genes underlying neuronal excitability, especially with respect to genes that may contribute to susceptibility to seizures though they cause no apparent seizure phenotype on their own.

Small mammals, especially mice and rats, have long been used as a model for epileptogenesis and epilepsy, most commonly through either electrically or chemically induced seizures. One well-known model is the classical kindling model, where repeated seizures are induced by chemical or electrical stimulus. This method added to knowledge about changes in behavior after recurrent seizures. There are other models of recurrent spontaneous seizures that begin with exposure to convulsants such as pilocarpine or kainate. There have also been studies of genetic and transgenic mouse seizure mutants that have been used to model specific types of epilepsy (reviewed in [10], [11]).

More recently, zebrafish studies have made progress in epilepsy research. Zebrafish have been used in anti-epileptic drug screening to test responses to chemically induced seizures while recording brain responses using electroencephalograms. There are also genetic zebrafish epilepsy models, such as one made by knocking down the voltage-gated potassium channel KCNQ3 using morpholinos (reviewed in [12]).

Drosophila is another model organism that has been actively involved in epilepsy research. In many ways, they are an ideal organism for particular aspects of this field of study, such as in mutant screens and anti-epileptic drug screens.

1.3 Drosophila as a model of seizure and epilepsy

The strengths of *Drosophila* as a genetic model organism make it an ideal system to use for epilepsy studies. Flies exhibit a number of interesting behaviors, including various seizure and paralytic phenotypes. Their short lifespans and small size make it possible to screen large numbers of flies quickly. The availability of genetic tools also allow for in-depth analysis of individual genes and interactions between them. Work in *Drosophila* has made many advances in characterizing seizure mutants and developing techniques that allow us to learn about their behavior, and then use these tools to explore the genetic basis of neuronal excitability and methods of seizure suppression.

1.3.1 Drosophila bang-sensitive mutants

Near the start of research into the study of neurogenetics in *Drosophila*, many behavioral mutants were collected in order to learn more about altered membrane physiology, often through changes in ion channel function. Some of the earliest known behavioral mutants that displayed seizure or paralysis phenotypes were indeed ion channel mutants. For example, three mutants were discovered that had leg-shaking behavior upon exposure to ether. These were named *Shaker* (*Sh*), *ether-a-go-go* (*eag*), and *Hyperkinetic* (*Hk*). The underlying cause of the phenotype was discovered to be defects in potassium ion channels. Likewise, three temperature-sensitive paralytic mutants, *temperature-induced paralysis* (*tipE*), *no action potential* (nap^{ts}), and *paralytic* (*para*) had defects in sodium ion channels (reviewed in [13]).

Bang-sensitive (BS) mutants have an even more dramatic seizure-like behavior compared to the leg-shaking and temperature sensitive mutants. Though

phenotypically normal in most circumstances, they respond to stimuli such as mechanical impact or electrical stimulus with a robust seizure and paralysis phenotype [13–15]. To date, there are 14 bang-sensitive mutants that represent 12 different genes [16]. One of the confusing aspects of these BS mutants was that the genes underlying these mutations code for a wide variety of gene products that do not have obvious connections despite their similar behavioral phenotypes. For example, the products of the genes affected in the BS mutants *technical knockout* (*tko*), *kazachoc* (*kcc*), and *couch potato* (*cpo*) are a mitochondrial ribosomal protein, citrate synthase, and RNA-binding protein, respectively [17–19]. *easily shocked* (*eas*) is a robust recessive BS mutant that is defective in an ethanolamine kinase, resulting in an altered membrane phosopholipid composition [20]. *slamdance* (*sda*) mutants have a defect in aminopeptidase N, which leads to an increased persistent sodium current in motoneurons, which suggests an increased excitability of the motor system. Electrophysiological and pharmacological tests indicate that the *sda* seizure phenotype may arise from defects in neural development [21], [22]. One of the most robust and difficult to suppress BS mutation is *paralytic*^{bangsenseless1} (*para*^{bss1}) which has been essential in developing a *Drosophila* model for seizure and epilepsy [23], [24]. Though this mutant was used as a model for seizure in flies for many years due to its tonic-clonic-like seizures, it was not until recently that *para*^{bss1} mutation was confirmed to be a gain-of-function allele of the sodium channel gene encoded by *para*. This gain-of-function mutation results in a defect of sodium channel inactivation [25]. It is also interesting to note that loss-of-function mutations in *para* are very effective seizure suppressors [24].

The seizure behavior of BS mutants follows a stereotyped pattern for most members of this group. After the mechanical or electrical stimulus, the flies have a brief seizure where all parts of their body, including wings, legs, proboscis, and abdomen, contract and shake. This brief seizure lasts only a few seconds and is followed by a period of paralysis where the flies are unresponsive to any

mechanical or electrical stimulus. Finally, the flies have a brief recovery seizure similar to the initial seizure [26]. *para*^{bss1} mutants deviate slightly from this pattern in that there is a tonic-clonic phase in between the period of paralysis and the recovery seizure [25]. Heterozygous BS mutants for the most part do not show BS behavior, except in the case of *sda* and *para*^{bss1}, which are semi-dominant. The penetrance of seizure is lowered greatly in *sda* heterozygotes, while *para*^{bss1} heterozygotes have higher penetrance. If seizure is initiated after the stimulus, these heterozygotes show typical BS seizures. In the case of *para*^{bss1} flies, this means that heterozygotes do not have a tonic-clonic phase in their behavior [25–27].

While the BS behavior is easily observed using a mechanical impact, this is an all-or-none behavior that can be only quantified by the penetrance of the BS phenotype, which is very high in homozygous BS mutants. In order to obtain more quantitative measurements of the change in seizure threshold in various BS mutants, a method of delivering electrical stimuli directly to the brain and eliciting seizure through the giant fiber (GF) pathway, which is responsible for the fly escape response, was developed. The activation of the GF pathway due to the electrical stimulus caused trains of seizure-like activity in the dorsal longitudinal muscles (DLM) of the flight muscles in BS animals [20]. During the paralysis phase of the seizure, there was a corresponding failure of the DLM to respond to any stimulation of the GF pathway [15]. Using this method, the seizure thresholds of various BS mutants were measured, allowing the mutants to be ranked by seizure susceptibility. Not surprisingly, *para*^{bss1} had the lowest seizure threshold, supporting its position as the most severe BS mutant [23].

1.3.2 Searching for suppressors and enhancers of seizure in Drosophila

With the availability of behavioral and electrophysiological techniques to quantify the seizure susceptibility of BS mutants, screens for possible genetic suppressors and enhancers of the BS phenotype became an area of interest.

Analysis of other mutations that have the ability to suppress or enhance the BS phenotype could facilitate the discovery of mechanisms or pathways underlying and regulating neuronal excitability. There is also the possibility of discovering novel targets for testing anti-epileptic and anti-convulsant drugs. In general, most of the discovered genes have been suppressors of BS mutations.

Some of the first experiments in this area observed the effects on different seizure and paralysis mutants in combination with each other. Therefore, these tests started with two behavioral mutants. Several of the temperature-sensitive paralytic mutants, such as nap^{ts} and *para*, were able to successfully suppress bang-sensitive seizures [26], [28].

Often a reverse genetics approach was taken in order to find enhancers and suppressors of bang-sensitivity. Genes known to affect neuronal excitability in other organisms are then tested in *Drosophila* to see their effects on seizure behavior. One example of this is the study of $shakB^{2}$. Previous work showed that mouse mutants of a specific connexin, or gap junction protein, had weakened epileptoform discharges in hippocampal slices [29]. Because of this result, $shakB^{2}$, a *Drosophila* gap junction protein mutant, was tested with BS mutants and the results indicated that this allele is able to suppress or partially suppress seizures in many BS mutants [30].

Genetic screens for suppressors of BS mutants have also been performed. Genes that are found in such screens are ones that have not previously been implicated in seizure susceptibility. For example, in a screen for gain-of-function seizure suppressors of *eas*, the gene *escargot* (*esg*) was identified multiple times as a seizure suppressor, among several other genes. *esg* is particularly of interest because it is involved in neural development and is able to suppress seizures in multiple BS mutants [31]. In another suppressor screen using P element mutagenesis in the *eas* background, the gene *topoisomerase I* ($top1^{JS}$) was

discovered to be a BS suppressor. This gene also had not been previously implicated in seizure susceptibility and is involved in *Drosophila* development [32]. Currently, there have been no large-scale screens searching for novel enhancers of seizure susceptibility in BS mutants.

1.3.3 Anti-epileptic drug screening in Drosophila

One of the greatest strengths of model organisms such as *Drosophila* is the ability to screen through many flies relatively quickly and without using excessive resources, such as in mutagenesis screens. With the availability of fly mutants that either cause seizure behavior or can suppress seizure behavior, screening for new anti-epileptic drugs in *Drosophila* becomes possible. Discovery of anti-epileptic drugs affecting BS mutants can also help reveal the mechanisms underlying the bang-sensitive phenotype, which is not well understood.

Much of the initial drug testing was to confirm that flies are able to respond to known anti-epileptic drugs in order to support *Drosophila* as a seizure model for human epilepsies. Anti-epileptic drugs that successfully suppressed BS seizures include gabapentin, phenytoin, valproate, and potassium bromide. Though these drugs were effective suppressors, other drugs tested were not able to suppress BS seizures [30], [33–36]. Some of the effects of these drugs include faster recovery from paralysis, or suppression of tonic-clonic behavior in $para^{bss1}$ [32].

Potentially novel anti-epileptic drugs have been suggested through testing inhibitors of $top1^{JS}$, a previously mentioned suppressor of BS seizures. Drugs that are known inhibitors of $top1^{JS}$ were tested on BS mutants. Some of these drugs, such as camptothecin and apigenin, were able to suppress BS behavior [32], [35]. This study represents the first example of discovery of potential anti-epileptic drugs through *Drosophila* BS mutants.

1.4 Perspectives

In this chapter, the genetics behind epilepsy and current work on *Drosophila* as a model for epilepsy were reviewed. While many aspects of *Drosophila* seizure susceptibility have been studied, such as the behavior of bang-sensitive mutants, the genes underlying these behaviors, and the ability of anti-epileptic drugs to suppress BS behavior, one area where there has not been much study are the genes responsible for seizure susceptibility, which can cause genetic predisposition for eventual epileptogenesis. In chapter 2, a screen for enhancers of the BS mutant *sda* is described, along with eleven candidate genes that may be enhancers of seizure susceptibility. In chapter 3, analysis of a gene, *pumilio*, which was found through this screen is described. This work is then summarized and discussed in chapter 4. Appendix A summarizes the results of the preliminary deficiency screen that took place prior to the screen described in chapter 2. Supplementary material for chapter 2 can be found in Appendix B, and supplementary material for chapter 3 is in Appendix C.

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CHAPTER 2

DEFICIENCY SCREEN FOR ENHANCERS OF THE BANG-SENSITIVE MUTANT *SLAMDANCE*

2.1 Abstract

Neuronal excitability is a key factor underlying the causes of disorders such as seizures and epilepsy and is greatly influenced by genetics. *Drosophila* is a useful system to study neuronal excitability and seizure susceptibility due to the large library of seizure and paralytic mutations available, as well as the ability to use high throughput genetic screening to look for new mutations. I am interested in finding genes that enhance neuronal excitability in flies using a sensitized seizure-susceptible background. Bang-sensitive (BS) mutants, in particular, display a robust seizure phenotype after a mechanical stimulus, and a subset of these mutants are semi-dominant and the seizure phenotype is only partially penetrant in heterozygous animals, allowing us to screen for enhancers that increase the severity or penetrance of the phenotype. Using the BS mutant *slamdance* (*sda*), I performed a deficiency screen with the Exelixis 3R deficiency kit, looking for deficiencies that increased the penetrance of seizure susceptibility. After identifying deficiencies that enhanced BS seizure penetrance when crossed to *sda*, I followed up with crosses to other deficiencies and alleles of genes within the deficiency to identify candidate BS enhancer genes. Using this method, I was able to find 10 genes with known functions that enhance seizure susceptibility, 8 of which have not previously been described to cause this phenotype.

2.2 Introduction

Epilepsy, a seizure disorder characterized by multiple unprovoked seizures, is extremely prevalent in the world today. It affects 1% of the overall population and is experienced by 3% of the population at some point in their lives [1].

Because epilepsy is so widespread, and affects all age groups, research for diagnostics and treatment are a high priority. While advances have been made in these areas for some epilepsies, knowledge in the area of epileptogenesis, or the process by which the brain becomes epileptic, is far from complete. Epilepsy can be caused by injury or illness, but this cannot account for all epilepsies. In these other cases, epilepsy is likely to be due to genetic causes. While pedigree studies of families with a high incidence of epilepsy have identified dominant mutations in genes that underlie epilepsies with a genetic basis, they can only explain a very small percentage of these genetically-based epilepsies. Known epilepsy-associated genes that encode ion channels in neurons, where mutations lead to channelopathies, can only account for less than 1% of all epilepsies [2]. Other genes associated with epilepsy are involved in very diverse biological processes, some of which previously had no connection to epilepsy or neuronal excitability [3].

In order to obtain a better understanding of known epilepsy genes, as well as discover new ones, we need to turn to model systems. Animal models, from insects to primates, have been useful in learning about epileptogenesis, such as through mouse models of electrical kindling or chemically induced seizures using convulsants [2], [4]. Certain models are also important for anti-epileptic drug screening [5]. However, one of the great strengths of model organisms is the greater ease in discovering mutations in genes that underlie the predisposition to seizure or epilepsy.

Drosophila in particular are suited for high throughput genetic screening and for determining gene function *in vivo*. Their short lifespans and a more compact genome with single copies of genes that may be found as multiples in a mammalian genome are two important features of this model system. The availability of well-developed genetic tools is also advantageous for screening or genetic manipulation. While this system has been essential in studying many

different biological processes, one concern may be that flies are not the ideal animal system to be studying a behavior such as epilepsy. However, there are many similarities between flies and mammals at the cellular level, such as in ion channels and membrane properties involved in synaptic transmission. There has been recent work showing that *Drosophila* can be used as a model for seizure in a number of different ways, including behavioral and electrophysiological analysis of existing seizure mutants, screening for genetic seizure suppressors, and searching for seizure-suppressing chemicals and drugs [6]. For example, electrical stimuli to fly brains can induce seizure-like behavior [7], [8]. Also, anti-epileptic drugs such as valproate [9], phenytoin, gabapentin [10], potassium bromide [11], and levetiracetam (Ronald Hoy and David Deitcher, personal communication) have been found to suppress seizure behaviors in flies.

Drosophila seizure mutants have altered neuronal excitability compared to normal flies and exist in several main categories: leg-shakers, temperature sensitives, and bang-sensitives [12]. The *Drosophila* bang-sensitive (BS) mutant class contains 14 alleles of 12 genes [6]. Some of these mutants, such as *paralytic*^{bangsenseless1} (*para*^{bss1}) and *slamdance* (*sda*), are semi-dominant in the heterozygous state [7]. *para*^{bss1} is considered to be one of the strongest bang-sensitive mutants, and the electrical activity in the brain during the seizure behavior is likened to tonic-clonic seizures. It is known to be a gain-of-function allele of the *paralytic* voltage-gated Na^+ channel [13]. Another bang-sensitive mutant, *sda*, has a weaker seizure phenotype than *para*^{bss1} [7]. The seizures in this mutant are due to a mutation in the Drosophila homolog of human aminopeptidase N, which causes an increase in persistent Na^+ current and reduced Ca^{2+} current [14], [15]. The semi-dominant phenotype in heterozygous *para*^{bss1} and *sda* manifests as a much lower penetrance of the seizure phenotype, meaning a smaller percentage of heterozygous flies have seizures in response to the stimulus than homozygous flies [7].

I wished to use this knowledge of bang-sensitive mutants to learn more about genes that may be involved in seizure susceptibility when mutated. My rationale for this is that there are many genes that may have not yet been found because their effect on bang-sensitivity is not apparent as a single mutation. Similar to how a variety of mutations can provide a genetic disposition toward seizure and epilepsy in people, yet seizures do not manifest unless another injury is present, there may be a number of genes in *Drosophila* that will reveal enhanced neuronal excitability only in the presence of another mutation providing the seizure-susceptible background. Much work has been done using bang-sensitive behavior to identify seizure suppressors [16–18], and these studies usually used the homozygous BS mutation in order to screen for suppressors. To screen for enhancers of BS seizures, I used the low penetrance of the heterozygous BS mutant as a sensitized background to find alleles of genes that may enhance this penetrance. The design of this screen is modeled on the *sevenless* screen which was used to discover components of the receptor tyrosine kinase pathway [19]. The rationale behind this particular screen was that a reduction of 50% of a pathway component can result in enhancement or suppression of the mutant phenotype. Although this was a very successful screen, one of the drawbacks was the time and effort needed to map the mutations created by EMS. With updated genetic tools available for *Drosophila* research, such as libraries of deficiencies with carefully documented endpoints, as well as thousands of transposon insertion mutants and RNAi lines, screening and mapping can be done much more efficiently. In order to understand more about genes that may affect bang-sensitivity directly as well as increase our knowledge base of genes that can enhance neuronal excitability as a heterozygous mutation, I have conducted an enhancer screen in a heterozygous bang-sensitive background to identify genes that, when mutated, increase the strength or penetrance of the bang-sensitive phenotype.

2.3 Methods

Fly stocks

All flies were raised on standard yeast and glucose media at 25°C. I used the Exelixis 3R deficiency kit (Bloomington Stock Center) for the deficiency screen. Several seizure mutants, specifically of the bang-sensitive class, were used in the heterozygous state as sensitized backgrounds for the screen. The three bang-sensitive lines I used include *slamdance* (*sda*), *paralytic*^{bangsenseless1} (*para*^{bss1}), and *easily shocked* (*eas*), and were a gift from Ronald Hoy. For behavioral controls, we crossed these bang-sensitive lines to w^{1118} (#5905 and #6326). Follow-up crosses to the screen involved deficiencies, transposon insertion stocks, or RNAi stocks available from the Bloomington Stock Center. A complete list of stocks used is found in Table B.1, and organized in greater detail in Tables B.5, B.6, and B.7. In cases of RNAi stocks, we targeted knockdown in all neurons using *elav*-GAL4 (#458 and #8763) recombined with *sda*.

Seizure behavior induction in adults

To test for bang-sensitive paralysis, we performed testing similar to that previously described [7]. Briefly, adult flies were collected 1-4 days post eclosion and anaesthetized with CO_2. Approximately 20 flies were placed in food vials with cotton plugs and allowed to recover for at least 12 hours before testing for penetrance of bang-sensitive paralysis. For seizure and paralysis induction, vials were turned upside down and given a mechanical vortex stimulus at maximum speed for 10 seconds (Vortex Genie 2, VWR Scientific). Immediately, the number of flies that were paralyzed or seizing were counted. Each vial was monitored for a minute for recovery of flies from paralysis in order to exclude flies that did not recover from seizure. All behavior testing was done during the same time of day, and approximately 60 flies of each genotype were tested. Data was represented as either a percentage of flies showing paralysis after vortexing over the total number of flies tested, or as a fold change of (% of flies paralyzed)/(% of control

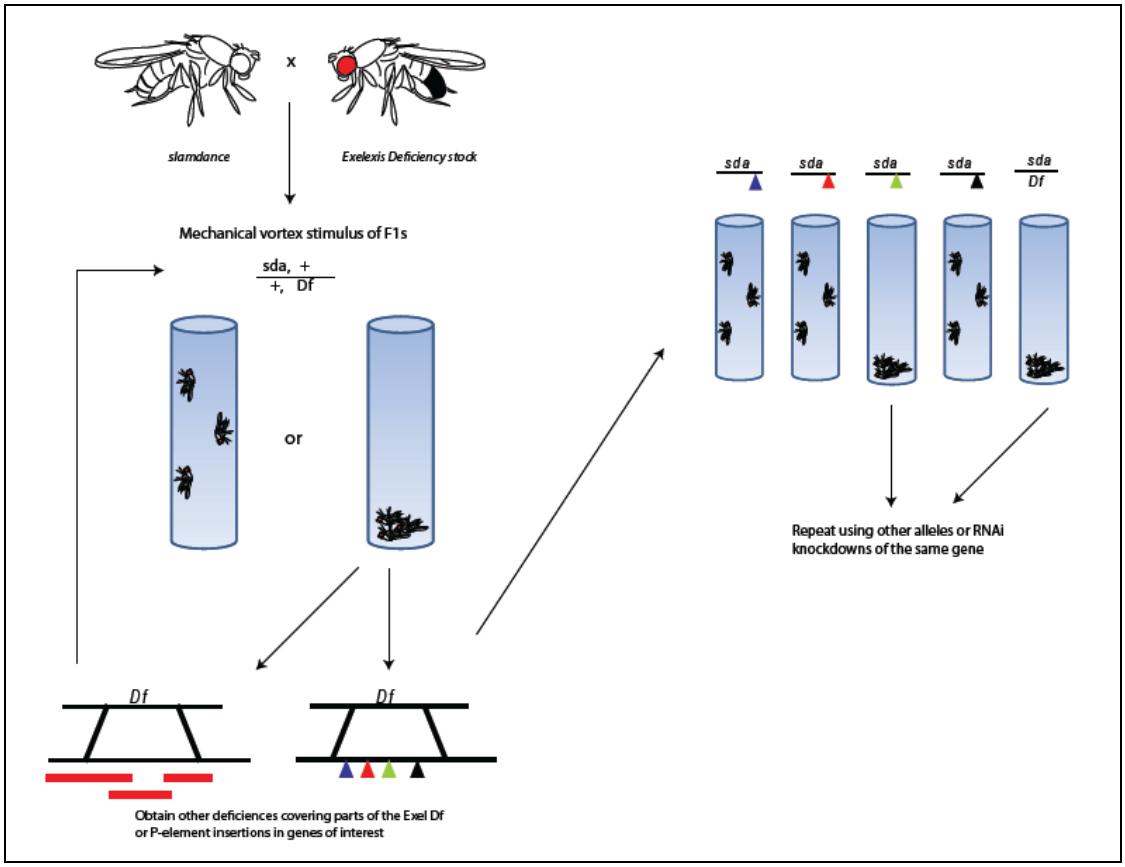


Figure 2.1. Schematic of deficiency screen for enhancers of *sda*

Bang-sensitive *slamdance* (*sda*) virgins were crossed to males of the Exelixis 3R deficiency kit and F1 progeny were tested for seizure susceptibility upon vortexing. Those lines that had a large increase in paralysis after vortexing compared to *sda*/+ were analyzed for genes of interest or other overlapping deficiencies. Further crosses testing overlapping deficiencies or alleles of genes of interest were done to determine putative enhancers of bang-sensitivity.

flies paralyzed). Controls were either *sda*/+ (to compare to all cases except for RNAi stocks) or *elav*-GAL4/+; *sda*/+ (to compare to RNAi stocks) flies.

Genetics

The deficiency screen for enhancers of bang-sensitivity was done as follows: *slamdance* virgins were crossed to males of the Exelixis Df 3R Kit and the F1 progeny were collected, sorted, and tested for the penetrance of bang-sensitive paralysis as described above. Deficiencies that caused an approximately twofold or greater increase in seizure penetrance compared to *sda*/+ controls were considered to contain a possible enhancer(s). Since there are available data for the endpoints of each Exelixis deficiency, the available data were used to identify overlapping deficiencies and transposon insertions or RNAi lines for genes within the Exelixis deficiencies. These lines were tested for bang-sensitive paralysis as described above.

2.4 Results

2.4.1 Deficiency screening for enhancers of bang-sensitivity penetrance

Within the category of seizure-susceptible Drosophila mutants, bang-sensitive flies have a particularly robust phenotype in that they display a stereotyped seizure-paralysis-seizure phenotype upon being given a stimulus, such as a mechanical impact [20]. Heterozygotes of some bang-sensitive mutants, such as *sda* and $para^{bss1}$ are semi-dominant and display a shorter seizure and paralysis phenotype at a lower penetrance than the homozygous mutant [7]. We used these characteristics of heterozygous bang-sensitive flies as a sensitized background in which to screen for genes that may be involved in neuronal excitability. Specifically, we crossed *slamdance* (*sda*) virgins to males of the Exelixis 3R Deficiency Kit and tested progeny from these crosses for bang-sensitivity, focusing on the number of flies displaying paralysis after vortexing (Figure 2.1). Our goal was to identify genes that showed non-allelic noncomplementation with *sda*, as this may uncover interactors with *sda* or other

Table 2.1
3R Exelixis deficiencies that enhance bang-sensitivity in *sda*

Exelixis Number	Stock Number	% paralyzed	Fold increase	Number tested	# of genes deleted
"Enhancers"					
Df(3R)Exel6150	7629	23.1	3.8	74	35
Df(3R)Exel6151	7630	63.3	10.4	120	2
Df(3R)Exel6154	7633	50.8	8.3	59	11
Df(3R)Exel6161	7640	23.9	3.9	71	15
Df(3R)Exel6165	7644	60.0	9.8	60	40
Df(3R)Exel6178	7657	21.7	3.6	60	48
Df(3R)Exel6196	7675	43.3	7.1	60	20
Df(3R)Exel6270	7737	71.7	11.7	60	23
Df(3R)Exel7379	7919	36.5	6.0	63	14
Df(3R)Exel7317	7932	40.4	6.6	52	17
Df(3R)Exel7305	7956	35.0	5.7	60	16
Df(3R)Exel8157	7973	32.4	5.3	71	9
Df(3R)Exel7328	7983	23.3	3.8	60	17
Df(3R)Exel9013	7991	23.3	3.8	60	15
Df(3R)Exel9025	7995	23.3	3.8	60	3
Control					
w1118	5905	6.1	1	180	1

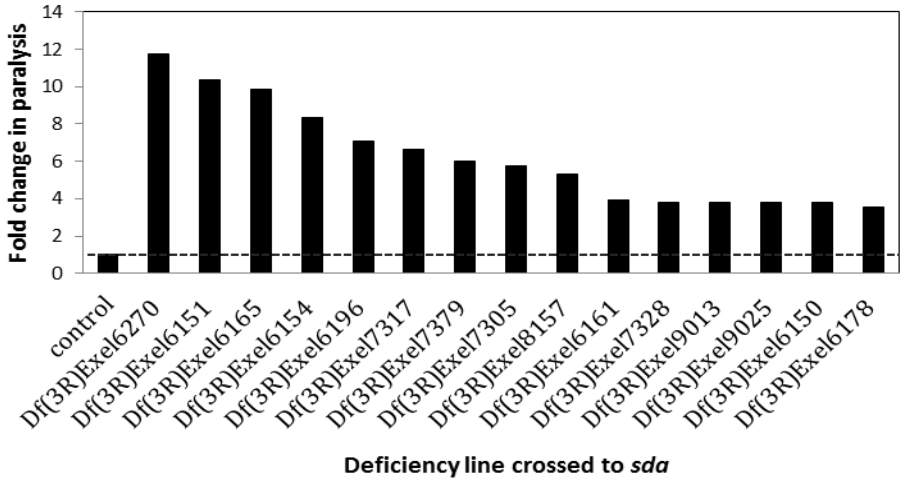
genes that generally affect the BS phenotype. The *sda* background was used over other bang-sensitive lines used because very few of the heterozygous *sda* flies show a bang-sensitive phenotype, while other bang-sensitives such as $para^{bss1}$ have much higher penetrance in the heterozygous state while *eas* heterozygotes are essentially wild-type in behavior (data not shown).

The Exelixis 3R Deficiency Kit covers approximately 65% of chromosome 3R over 138 deletion lines [21] and we began the screening process with this set of stocks. After crossing the deletion lines of this kit to *sda* and behavior testing each set of progeny (A complete list of all stocks tested and behavioral results can be found in Table B.2), we discovered 19 deficiencies that, when crossed to *sda*, caused greater than 20% of the F1 progeny to seize upon vortexing (Table 2.1). Paralysis of 20% of the flies tested represented a 3.8 times fold increase in paralysis compared to the *sda*/+ control. Four of the 19 deficiencies were removed from the list since other deficiencies that were not enhancers completely overlapped these four, leaving 15 deficiencies containing potential enhancers of the BS phenotype.

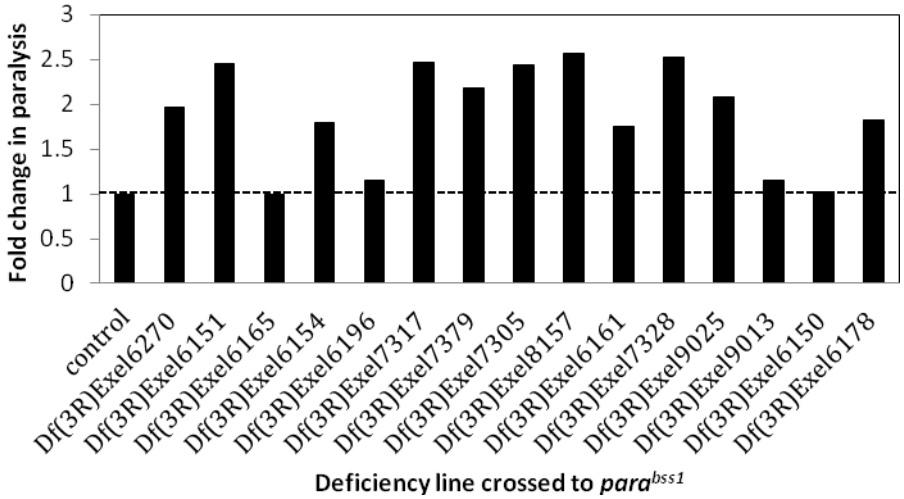
We investigated the specificity of the enhancement of seizure susceptibility to the *sda* background by crossing each of the fifteen Exelixis deficiencies to two other bang-sensitive lines, $para^{bss1}$ and *eas.* We hypothesized that some of these deficiencies would cause enhancement of bang-sensitivity only in *sda*, possibly as part of a *sda*-specific pathway, or alternatively may be a general enhancer of neuronal excitability to several bang-sensitive lines. The F1 progeny from these crosses were tested as described for *sda*, though only females were tested as both $para^{bss1}$ and *eas* are X-linked. For $para^{bss1}$, four of the fifteen sets of F1 progeny resulted in seizure susceptibility relatively unchanged from that of control $para^{bss1}$/+ flies, while the other lines had an increase in flies showing paralysis (Figure 2.2 B, Table B.3). The maximum fold change of these tested flies is lower than that observed with *sda* (2.6-fold for $para^{bss1}$ compared to 11.7-fold for *sda*)

Figure 2.2 Seizure penetrance enhancement by Exelixis deficiencies in three bang-sensitive backgrounds. (A) Fold change in paralysis in a *slamdance* background, (B) in a *para*^{bss1} background, and (C) percent paralyzed in an *easily shocked* background. N≥40 for each cross.

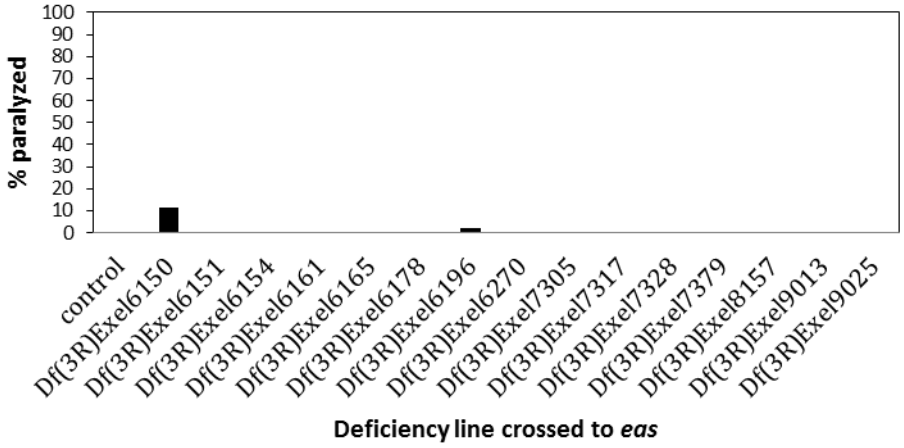
A *sda*



B *para*^{bss1}



C *eas*



due to the higher penetrance of bang-sensitivity in the *para*^{bss1}/+ background. *eas*, on the other hand, is a recessive mutation and *eas*/+ females are not bang-sensitive. Crossing *eas* to the 18 deficiencies produced no increase in bang-sensitivity except for a very few flies showing seizure behavior in two lines, Exel6151 (4/52) and Exel6270 (1/89), which were the strongest two enhancers for *sda* (Figure 2.2 C, Table B.4).

2.4.2 Searching for genes involved in enhancing neuronal excitability

The Exelixis deficiency library is suited for enhancer and suppressor screening due to each deficiency having well-documented breakpoints and lists of genes deleted within the deficiency [21]. Once we determined the eighteen deficiencies that enhanced bang-sensitivity, we followed up first by searching for existing deficiencies that overlap the Exelixis deficiencies in order to narrow down the window of candidate genes. These follow-up deficiency tests are summarized in Figure B.1 and Table B.5, though some of the Exelixis deficiencies do not currently have any overlapping deficiency lines available for testing.

Following this step, we searched for existing alleles of candidate genes within the Exelixis deficiencies to test with *sda*. We focused on genes highly expressed in the brain and the nervous system relative to the rest of the expression pattern, either in larval or adult stages. These genes would be the most likely candidates for direct involvement in neuronal excitability instead of resulting indirectly through other defects. We preferentially selected available null alleles of the candidate genes as they would be most similar to a deletion, though in some cases only hypomorph alleles were available. These alleles were crossed to *sda* and tested for bang-sensitivity, summarized in Figure 2.3 and Table B.6.

For some candidate genes, instead of mutant alleles, we obtained RNAi lines to test the effect of knockdown of the gene on bang-sensitivity. Since we were searching for candidate genes highly expressed in the nervous system, we tested

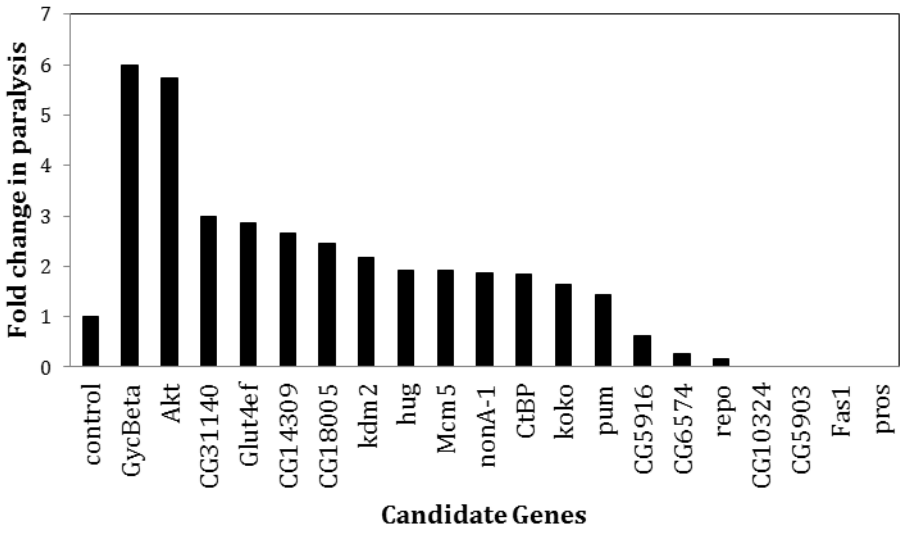


Figure 2.3 Effects of transposon insertion alleles in enhancing bang-sensitivity penetrance. P-element insertion alleles of potential bang-sensitive enhancer were crossed to *sda* and progeny were vortex tested. N ≥ 60 flies per gene, except *Akt* (N=20), CG10324(N=34), and CG5903 (N=20).

these RNAi lines by crossing males to *elav*-GAL4; *sda* females to cause panneuronal knockdown of the gene. We tested the F1 progeny using the same behavioral protocol as in the screen and used *elav*-GAL4/+; *sda*/+ as a behavioral control (Figure 2.4, Table B.7). In general, crosses with an *elav*-GAL4; *sda* background tended to have lower seizure penetrance than crosses in a *sda* background

A summary of potential enhancer genes for *sda* is provided in Table 2.2 and a more detailed table summarizing enhancer Exelixis deficiencies and all follow-up crosses for each Exelixis line is found in Table B.10.

2.4.3 Deficiencies lethal in combination with sda

In addition to deficiencies that enhanced seizure susceptibility, we found two Exelixis deficiencies that showed partial synthetic lethality in combination with *sda* but not with $para^{bss1}$ and *eas* (Table 2.3). To determine the genes underlying the lethality, we obtained overlapping deficiencies and several alleles of candidate genes to cross and test for viability. The cross between Exel6263 and *sda* produced very few flies with the genotype Exel6263/*sda*. Three overlapping deficiencies were tested and we were able to narrow down the window of candidate genes to a region of approximately 6 Mbp containing 12 genes (Table 2.4, Figure B.2. Candidate genes can be found in Table B.8).

The second deficiency, Exel6214, crossed to *sda*, produced a low number of Exel6214/*sda* progeny compared to the balancer siblings (14/80 total) instead of the expected 50% of the total progeny. We tested three genes within this deficiency with *sda* to see if any of these might be the cause of the partial lethality. While the genes tested did not cause lethality (Table B.9), one of these, *similar* (*sima*), caused a large enhancement in bang-sensitivity when crossed to *elav*-GAL4; *sda* (Figure 2.4). However, tests of a deficiency overlapping *sima* (Df(3R)BSC502, #25006) did not confirm this enhancement in

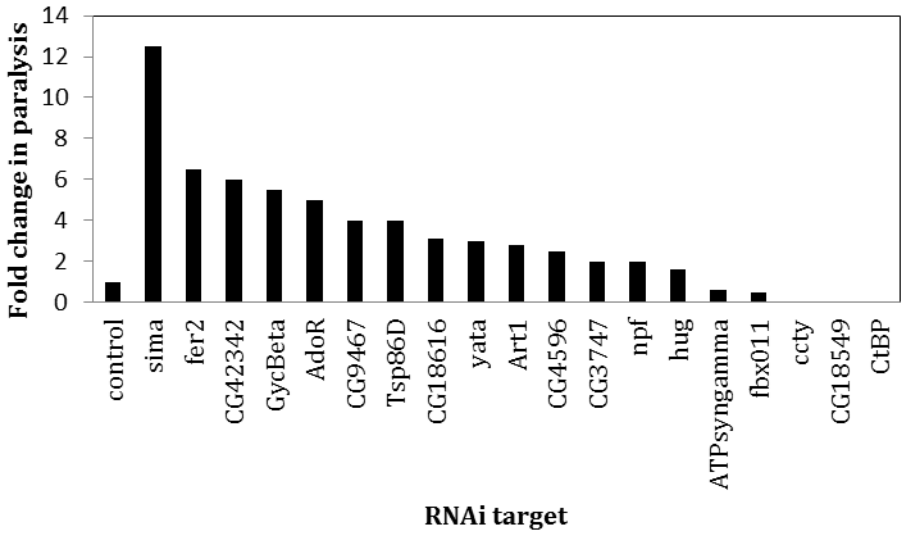


Figure 2.4 Effects of neuron-targeted RNAi knockdown in enhancing bang-sensitivity. RNAi targeted to all neurons by crossing *elav*-GAL4; *sda* to UAS-RNAi constructs of putative *sda* enhancers. Data represented as fold change as compared to control (*elav*-GAL4/+; *sda*/+). N≥69 per RNAi line, except for CG18616 (N=13) and *ccty* (N=17).

bang-sensitivity, though the larger knockdown provided by RNAi targeted only to neurons may explain the difference in behavior and viability.

2.5 Discussion

2.5.1 Deficiency screen identifies genes that are able to enhance seizures

At the time when this screen was designed and a preliminary set of deficiencies was tested, our current knowledge of the extant Drosophila seizure excitability mutants showed a wide array of genes with disparate functions and no clear relationship between those with very similar behavioral phenotypes, such as the BS mutants which all displayed a stereotypical seizure-paralysis behavior after a mechanical stimulus [22]. For example, the gene product of *sda* is an aminopeptidase [14], the gene product of *eas* is an ethanolamine kinase [23], and the product of another BS mutatione *technical knockout* (*tko*) is a mitochondrial ribosomal protein [24]. We performed this deficiency screen using one of these BS lines, *sda*, in order to find interactors that may help us to elucidate a pathway that might underlie the seizure phenotype, and perhaps link the different BS lines together.

Using *sda* as the sensitized seizure background, the Exelixis 3R kit, and mechanically induced seizures, we found 19 Exelixis deficiencies that enhanced the BS phenotype of *sda* heterozygotes. With further testing using available deficiencies, transposon insertion lines, and UAS-RNAi lines, we were able to identify 13 genes as candidate enhancers of seizure susceptibility. The products of 11 of these genes have known functions that range in a variety of processes.

Only one of the enhancer genes found in this screen, CG9467, encodes a protein directly associated with an ion channel. Though not much is known about CG9467, the gene has homology to the human epilepsy gene KCTD7 (potassium channel tetramerization domain-containing), which functions in regulating potassium conductance in neurons [25]. It is also involved as an auxiliary

Table 2.2
Candidate enhancers of *sda* bang-sensitivity

Exelixis Deficiency	Candidate Enhancer CG No.	Gene name	Function	Biological Processes
Exel6151	CG9755	*pumilio* (*pum*)	translational repressor	synaptic transmission dendrite morphogenesis pole cell migration
Exel6154	CG9467		oxidoreductase activity	oxidation-reduction process potassium ion transport
Exel6178	CG14309		unknown	unknown
Exel6196	CG31140		diacylglycerol kinase activity	lateral inhibition
Exel6214	CG7951	*similar* (*sima*)	signal transducer activity	positive regulator of transcription
Exel6270	CG42342		unknown	unknown
Exel7305	CG4591	*Tetraspanin 86D* (*Tsp86D*)	Cell adhesion, signal transduction	nervous system development
Exel7317	CG6359	*Sorting nexin 3* (*Snx2*)	phosphatidylinositol binding	Wnt receptor signaling pathway
	CG18616		DNA-binding transcription factor	DNA-dependent regulation of transcription
Exel7328	CG4006	*Akt1*	protein serine/threonine kinase	many developmental processes
	CG5952	*48 related 2* (*fer2*)	DNA-binding transcription factor	neuron development locomotor activity
Exel7379	CG1470	*Guanylyl Cyclase β* (*Gycβ*)	guanylate cyclase activity	cGMP processing
Exel9025	CG1973	*yata*	protein kinase	protein phosphorylation

subunit of $GABA_B$ receptors in which KCTDs are able to affect the kinetics of the $GABA_B$ receptor [26].

The other nine genes found in this screen are not associated with channelopathies. However, since our candidate gene search was biased towards those that have significant expression in the nervous system, most of these genes are present at moderate to high levels in brain or central nervous system, so it is possible that many of these enhancer genes have a function in the nervous system. Some of the biological function categories that these genes fall under include neural development, translational control, secretory pathway, and others.

2.5.2 Genes affecting neural development

Recently, work on *sda* has shown that the seizure phenotype is due to increased persistent Na^+ current in the central neurons as well as changes to Ca^{2+} and Na^+ conductances in motoneurons of *sda* larvae. The role of the aminopeptidase in causing this increase of Na^+ current is still unknown, though it is hypothesized that the change in Na^+ currents and ion conductances alters neural development [15]. It is possible that in the presence of other mutations that affect neural development, the effects of *sda* activity on nervous system development might be exacerbated.

One of the genes found in this screen, CG31140, is a diacylglycerol kinase that may be involved in neural glial development through lateral inhibition. This gene is downregulated in the *glial cells missing* mutant, where glia are transformed into neurons [27]. Interestingly, this is the only gene from our screen that appears to enhance BS seizure in the *sda* heterozygote but not the $para^{bss1}$ heterozygote. *Tetraspanin 86D* (*Tsp86D*) is another enhancer found in the screen. This gene encodes a transmembrane protein expressed throughout the nervous system and is known to be required for normal formation of the ventral nerve cord and proper axon guidance [28], [29]. Another gene, *48 related 2* (*fer2*), is a

Table 2.3
3R Exelixis deficiencies that show partial synthetic lethality in combination with *slamdance*

			slamdance	*para*^{bangsenseless}	*easily shocked*
Exel6263/TM6B, Tb (#7730)	Exel6263	female	0	46	35
		male	5	49	18
	TM6B, Tb	female	43	33	23
		male	37	20	21
Exel6214/TM6B, Tb (#7692)	Exel6124	female	11	52	53
		male	3	42	32
	TM6B, Tb	female	38	39	50
		male	29	36	21

transcription factor required for the development of ventral lateral neurons, which are known as pacemaker neurons for circadian locomotor rhythms [30].

2.5.3 Genes affecting the secretory pathway

Neurons are highly reliant on the secretory pathway for normal function. Packaging, transporting, and releasing neurotransmitters and peptides are all carefully regulated and involve many components. One of the BS enhancer genes, *yata*, is believed to be involved in the sorting of vesicles in regulated secretory pathways, such as those needed for synaptic transmission [31]. Another study of *yata* suggests that it is needed for regulation of protein localization, specifically the amyloid precursor-like (APPL) protein in *Drosophila* [32]. Another BS enhancer and secretory pathway gene, *Sorting nexin 3* (*Snx3*), is a protein expressed in cytoplasm and is a part of the retrograde transport from endosomes to the trans-golgi network [33]. Though they both are able to increase seizure susceptibility in BS heterozygotes, the method by which these genes cause this is unknown.

2.5.4 Genes affecting O_2 and NO detectors

Sima is the *Drosophila* version of mammalian *hypoxia inducible factor α* (*HIF-α*) and is regulated by oxygen levels by a protein domain that functions as an oxygen sensor [34]. The normal function of Sima is to detect hypoxia, which leads to the cell's response to low oxygen conditions [35]. In the case of the BS enhancer screen, it is possible that the knockdown of Sima due to RNAi renders cells unable to respond to low oxygen levels during or after a seizure, leading to an increase in severity.

Similarly, the BS enhancer gene *guanylyl cyclase β* (*gycβ*) is a subunit of a nitric oxide (NO) sensor in the brain [36]. NO is able to function as a signaling molecule and as a neurotransmitter, and is reported to have a connection with human epilepsy, though various studies have given mixed results (reviewed in [37]).

Table 2.4
Follow-up deficiency testing for lethality caused by Exel6263

Deficiency	Stock Number	Breakpoints	Viability when crossed to *sda*
Df(3R)Exel6263	7730	84E6--84E13	Lethal
Df(3R)BSC196	9200	84E6--84E11	Lethal
Df(3R)BSC196	9622	84E6--84E8	Lethal
Df(3R)BSC222	9699	84E8--84F6	Viable

gycβ along with α subunits regulates 2nd messenger pathways that result from the presence of NO due to neural activity or stressors [38]. Work has shown that *gycβ* may be able to use the presence of NO due to neural activity to detect coincidence of signals received by a neuron [39].

2.5.5 Translational control and neuronal excitability

Translational control is essential for gene regulation during all stages of development, often through mechanisms such as polyadenylation and deadenylation, recruiting 4E binding proteins, repression by microRNAs, and ribosomal binding regulation (reviewed in [40]). Studies in *Drosophila*, *C. elegans*, and *Xenopus* have shown that translational control is required for oogenesis and early development [41], [42]. However, evidence suggests that translational control is connected to neuronal excitability as well, such as in regulating neural development (reviewed in [43]). Therefore, it is not surprising that several of the genes, specifically *Akt*, *pumilio* (*pum*), and CG18616, found in this screen are involved in translational control. *Akt* is a part of a pathway responsible for elF2α phosphorylation in response to stress. The end product of this pathway is the inhibition of mRNA translation [44]. CG18616 is a transcription factor that is the fly homolog of human NOT10, which is a part of the carbon catabolite-repression 4-NOT (CCR4-NOT) complex involved in deadenylation of mRNA [45]. Though not much is known about CG18616 itself, the CCR4-NOT complex is suggested to be a "chaperone platform" present in most parts of the organism that helps to regulate and link multiple proteins that are involved in different mechanisms of translational control [46].

Of the genes found in the screen, *pum* was the only gene previously reported to increase neuronal excitability. Pum is a translational repressor best known for its function in early development where it is implicated in maintenance of germline stem cells and establishing the anterior-posterior axis through regulating expression of *hunchback* [47], [48]. More recent work has shown that

pum regulates neuronal excitability. Specifically, Pum is involved in the formation of higher order dendrites [49]. This function in dendritogenesis is also supported by evidence from the mammalian homolog of *pum*, *Pumilio2* [50]. Another study showed that levels of *pum* mRNA is regulated by amounts of neuronal activity, and that lowering levels of Pum itself increased the mRNA levels of a voltage-gated Na^+ channel [51]. Therefore, it was not surprising to identify *pum* as an enhancer of seizure behavior. It is interesting to note that many genes that are associated with *pum*, either by functioning in the same translational regulatory pathway, or by being regulated by Pum, have also been connected to neuronal excitability [49], [52], [53] and suggests that further study of *pum* and associated genes using this screening method may result in discovery of more enhancers of BS seizures.

2.5.6 Deficiency screening for enhancers of bang-sensitivity

Our genetic screen represents one of the few to search for enhancers of seizure susceptibility using a BS mutant as the sensitized background. We expected to find both general enhancers of seizure as well as possible interactors of the neural membrane aminopeptidase encoded by *sda* [14]. Through this screen, we identified 12 known genes, excluding *pum*, that were previously not connected to any phenotype related to enhancing neuronal excitability or seizures. The deficiency screening method allowed for relatively quick forward genetic screening of specific regions of the genome followed up by a reverse genetics approach of determining if mutations of specific genes can increase BS behavior. Enhancer screens typically identify components of pathways [54] but the genes we identified may not be specific enhancers of *sda* as they enhanced multiple bang-sensitive lines, though further testing is needed to confirm this. One interesting result is the finding of two deficiencies that appear to be semi-lethal in combination with *sda*. Though the identity of the genes that cause this synthetically lethal phenotype is unknown, they may have a more direct connection to *sda* function.

Although this screen only covered 65% of chromosome 3R, we believe this method can be effectively extended to encompass most of the *Drosophila* genome since the combination of Bloomington Deficiency Project, Exelixis, and the DrosDel deficiency collections together cover between 94-98.9% of genes on each chromosome. The initial stage of the screen is quick and efficient due to needing only one generation of crosses, the follow-up stages can be more time consuming since a large collections of stocks need to be obtained for the subsequent testing. This was one of the reasons that our search for enhancer genes was biased towards those with neuronal expression, in order to narrow down our search pool of genes as much as possible.

One note to make about this screening method is that many of the deficiencies that were found to enhance BS phenotype penetrance gave a much higher percentage of flies seizing than did any of the individual enhancer genes. This can be due to the deficiency causing an entire deletion of the gene, while the alleles could be hypomorphs, but this can also be due to multiple genes within the deficiency that contributing to the BS phenotype. For example, alleles of *pum* never enhanced the BS phenotype as much as the Exelixis deficiency containing *pum*. However, the deficiency also contained two other genes that slightly enhanced the seizure penetrance, and it may be possible that these genes in combination with *pum* allowed the Exelixis deficiency to give a stronger phenotype. In future iterations of this screen, it may be important to consider other genes that enhance the BS phenotype instead of selecting only the strongest one.

In summary, in this work we screened for genes, which when mutated or knocked down, enhance the penetrance of the BS seizure phenotype. The genes found in this work encode a range of different products such as kinases, transcription factors, and translational repressors, among others. Though they

mostly have unrelated functions, their effects on enhancing bang-sensitivity are almost identical, varying only in the actual magnitude of seizure penetrance. Most of these genes enhanced at least two BS lines, indicating that they may contribute to a general increase in bang sensitivity, though one gene, CG31440, has a specific effect on *sda*. Since our screen generally focused on genes that were expressed at moderate to high levels (according to FlyAtlas Anatomical Expression Data) in the nervous system, a large number of the genes we identified as enhancers have known roles in the nervous system, though none have been directly implicated in seizure behavior or increases in neuronal excitability. Further work on these genes alongside the BS mutants may help us to understand how mutations in these genes can lead to increases in neuronal excitability.

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CHAPTER 3

THE TRANSLATIONAL REPRESSOR *PUMILIO* IS NECESSARY DURING NERVOUS SYSTEM DEVELOPMENT FOR NORMAL NEURONAL EXCITABILITY IN ADULT *DROSOPHILA*

3.1 Abstract

The gene *pumilio* (*pum*) encodes a translational repressor well known in *Drosophila* development to regulate anterior-posterior patterning and germ line maintenance. More recent work has implicated this gene in later stages of development, specifically in the nervous system, to regulate processes such as expression of ion channels and dendrite arborization. Studies in other organisms also support the function of *pum* in neuronal development and excitability. We discovered that *pum* is also able to enhance seizure susceptibility in a sensitized background when mutated, and therefore we have used this phenotype to learn more about the timing and location of Pum function with relation to neural development. Using behavioral tests, electrophysiology, and immunohistochemistry, we show that the effect of *pum* mutation on neuronal excitability is greatest when there is a reduction of Pum levels in cholinergic and GABAergic neurons, in comparison to other types of neurons, and that this reduction is required at multiple stages of development.

3.2 Introduction

Translational control is an essential component of development in many organisms and affects the regulation of a wide range of mRNAs through various mechanisms (reviewed in [1]). Much work has been done in showing the role of translational control in maintenance of germ cells and in the cell cycle, especially in *Drosophila* and *C. elegans* [2]. However, increasing evidence reveals the importance of translational control in the normal development and functioning of

the nervous system [3], [4] by providing a means of post-transcriptionally regulating gene expression to specific locations where the translation of the mRNA is needed. Since neurons are specialized cells and highly polarized, it is essential that gene expression is regulated at specific sites of the cell to ensure proper development. Also, since neurons are compartmentalized and often have axons and dendrites that may extend away from the cell body, regulating mRNAs already present in these structures provides a fast method of controlling gene expression, instead of using *de novo* mRNA synthesis [5], [6].

One example of translational control by a gene that is essential both in early embryogenesis and in neural development is that of the gene *pumilio* (*pum*). In Drosophila, Pum protein functions as a translation repressor as a part of the PUF RNA-binding protein family and has orthologs in worms [7], plants [8], *Xenopus* [9], mice [10], and humans [11]. Pum is known to have a role in regulating many functions, and has been well characterized in early stages of the Drosophila life cycle, including establishment of the anterior-posterior axis, abdominal segmentation [12], and maintenance of germline stem cells [13]. Early on, the main focus of *pum* research was on its role regulating embryonic anterior-posterior axis formation through several genes such as *hunchback*. However, a new mutant, named *bemused*, was found as a behavioral mutant with reduced coordination and flight ability [14] and was eventually discovered to be an allele of *pum* [15]. From this point, a number of discoveries were made on the involvement of *pum* in neuronal excitability.

As Pum was revealed to be involved in several adult processes, many efforts were made to discover its targets and gain a more thorough understanding of its functions [16], [17]. Pum is believed to have a wide range of targets for repression, as the consensus mRNA binding sequence for Pum is found in over 1000 genes [16]. Furthermore, the mRNAs known to be localized to the synapse were screened for *pum* mRNA binding by using computational, biochemical, and

in vivo assays [17]. A screen searching for genes involved in learning and memory, by both mutagenesis and microarray, revealed the gene *pum* as well as *staufen*, a gene which has a Pum binding sequence in its mRNA, suggesting Pum's role in synaptic modification [18]. More evidence for this came from another study showing that the translational control by Pum does indeed play a role in synaptic modification, specifically in the elaboration of higher order dendrites [19]. This was followed up by work on *pum* in the mouse model showing that microRNAs regulate levels of Pum in order to control dendritogenesis [20].

More direct evidence that *pum* is involved in synaptic transmission came from another study when the *pum* allele, $pum^{bemused}$, crossed into a seizure-susceptible background showed that levels of *pum* mRNA itself are regulated by neuronal activity. Reduction in Pum resulted in an increase of *paralytic* (*para*) mRNA, which encodes a voltage-gated Na^+ channel. Likewise, overexpression of Pum caused reduction in the magnitude of the Na^+ current in motoneurons [21]. However, while there is evidence connecting *pum* to functions in synaptic transmission and neuronal excitability, its expression pattern in the adult nervous system, the effects of *pum* mutations on the behavior of seizure-susceptible mutants, and the importance of Pum in specific stages of development for normal neuronal excitability remains unknown.

To better understand the role of *pum* in regulating neuronal excitability, we investigated its effect on seizure behavior as well as its expression in the larval and adult nervous system. Behavioral analysis revealed that a decrease in Pum due to mutation increases the penetrance of seizure in a sensitized bang-sensitive (BS) background. This increase in BS seizure was apparent even when the Pum allele is heterozygous. We show that this seizure behavior increase correlates with a lowering of the voltage of the stimulus required to directly induce a seizure. In analyzing the expression pattern of Pum, we found that all cells positive for Pum also are positive for neural markers, indicating that the protein

is present only in neurons. Panneural knockdown of Pum using RNAi reduces most of its expression in the brain. Targeted knockdown of Pum specifically in cholinergic and GABAergic neurons produces a strong increase in seizure behavior as well. Finally we show that reduction of Pum in both larval and adult stages is needed in order for the increase in BS seizure behavior to be apparent. Our results show that the function of Pum in neurons during development and adult stages is necessary for normal neuronal excitability at the adult stage.

3.3 Methods

Fly stocks

Flies were maintained on standard yeast and glucose media at 25°C. Bang-sensitive stocks *slamdance, easily shocked,* and *para*^{bangsenseless} were a gift from Ronald Hoy. Except when mentioned, all other stocks are available from the Drosophila Stock Center (accompanied by stock number). *w*^{1118} (#5905 and #6326) crossed to bang-sensitive stocks were used as a behavioral control. Deficiencies used that affect pumilio include, *Df(3R)by10, red*^{1} *e*^{1}*/TM3, Sb*^{1} *Ser*^{1} (#1931), *w*^{1118}*; Df(3R)Exel6151, P{w*$^{+mC}$*=XP-U}Exel6151/TM6B, Tb*^{1} (#7630), *w*^{1118}*; Df(3R)Exel6152, P{w*$^{+mC}$*=XP-U}Exel6152/TM6B, Tb*^{1} (#7631), and *w*^{1118}*; Df(3R)BSC666/TM6C, Sb*^{1} *cu*^{1} (#26518). *pumilio* alleles used include *w***; P{w*$^{+mC}$*=lacW}pum*^{bem}*/TM6* (#6782), *P{ry*$^{+t7.2}$*=PZ}pum*^{01688} *ry*^{506}*/TM3, ry*^{RK} *Sb*^{1} *Ser*^{1} (#11544), and *y*^{1} *w*^{67c23}*; P{y*$^{+t7.7}$ *w*$^{+mC}$*=wHy}pum*^{DG05207}*/TM3, Sb*^{1} *Ser*^{1} (#21042).

In tests using knockdown of pumilio, we crossed a pum-RNAi line (pum-RNAi, #101399, Vienna Drosophila RNAi Center) to various GAL4 lines. For tests requiring overexpression of pumilio, we crossed UAS-*pum* (gift of Michael Stern) to various GAL4 lines. For specific temporal control of pumilio RNAi, we recombined pum-RNAi with a Gal80^{ts} line, *w***; P{w*$^{+mC}$*=tubP-GAL80*^{ts}*}10; TM2/TM6B, Tb*^{1} (#7108) to be used with GAL4 lines. Likewise, for temporal control of UAS-*pum*, we recombined UAS-*pum* with the same Gal80^{ts} line.

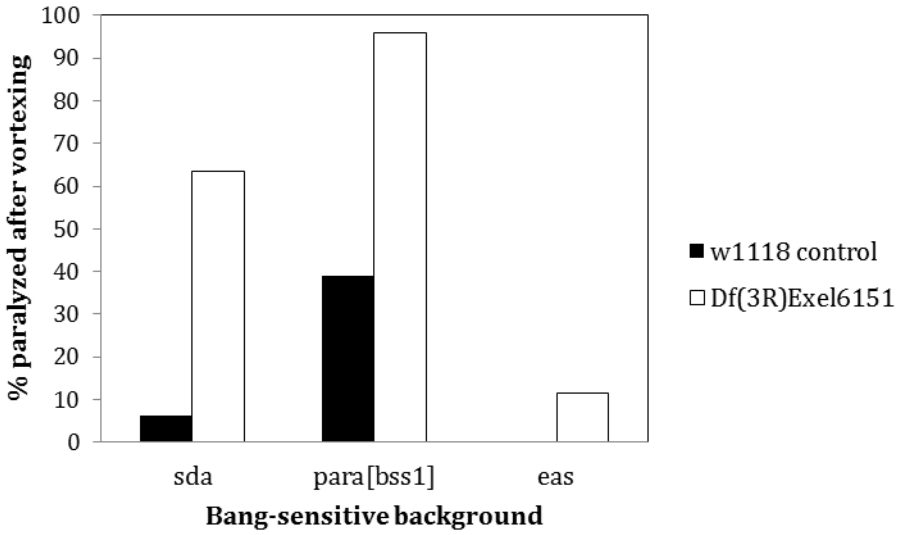


Figure 3.1 Exel6151 enhances bang-sensitivity penetrance. Exel6151 deficiency was crossed to three bang-sensitive lines and tested for seizure penetrance. N≥60 for each genotype.

GAL4 lines used include promoters of the following genes: *Choline acetyltransferase* (*cha*) (w^{1118}; *P{w*$^{+mC}$*=Cha-GAL4.7.4}19B/CyO, P{ry*$^{+t7.2}$*=sevRas1.V12}FK1*) (#6798) and *Vesicular acetylcholine transporter* (*VAChT*) (w^{1118}; *P{GMR55G09-GAL4}attP2*) (#46075) to target cholinergic neurons, *vesicular glutamate transporter* (*P{w*$^{+mC}$*=VGlut-GAL4.D}1, w**) (#24635) to target glutamatergic neurons, *reversed polarity* (w^{1118}*; P{w*$^{+m*}$*=GAL4}repo/TM3,* Sb^{1}) (#7415) to target glia, *β amyloid protein precursor-like* (*P{w*$^{+m*}$*=Appl-GAL4.G1a}1,* y^{1} *w**) (#32040) to target neurons, *tyrosine decarboxylase* (*w***; P{w*$^{+mC}$*=Tdc2-GAL4.C}2*) (#9313) to target octopaminergic neurons, *Actin* (y^{1} w^{*}; P{Act5C-GAL4-w}E1/CyO) (#25374) to target all cells, *slit* (*w***; P{w*$^{+mC}$*=GAL4-sli.S}3*) (#9580) to target midline glia, *elav* (*P{w*$^{+mW.hs}$*=GawB}elav*^{C155}) (#458) to target neurons, and *Gad2B-GAL4* (gift of Leslie Griffith) to target GABAergic neurons.

Larval Immunohistochemistry

Wandering third instar larvae were dissected in cold HL3.1 without Ca2+ and fixed in 4% paraformaldehyde for 30 minutes. Larvae fillets were washed three times quickly and three times 15 minutes in PBS + 0.3% Triton-X + 10 mM Tris HCl pH 7.5 (PBST) and then blocked in 10% donkey serum for one hour at room temperature. The larvae were incubated in primary antibody overnight at 4°C, then washed again in PBST three times quickly and three times 15 minutes. After this, the larvae were put in secondary antibody in PBST + 10% donkey serum incubation for four hours at room temperature, and then washed again in PBST wash solution, at which point they were mounted on a glass slide using Vectashield (Vector Labs) which are then stored in the dark at 4°C.

Primary antibodies were used as follows: Anti-pumilio A (rat, 1:1000, gift from Paul MacDonald), anti-elav (mouse, 1:100, Developmental Studies Hybridoma Bank), anti-βgal (mouse, 1:200, Promega), anti-GFP (rabbit, 1:2000, Invitrogen). Secondary antibodies are as follows: goat anti-rat 488 (Invitrogen), goat anti-

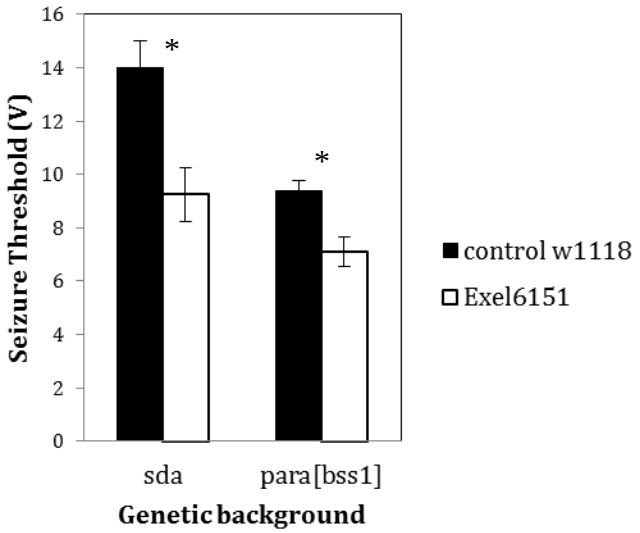


Figure 3.2 Seizure threshold in bang-sensitive heterozygotes reduced by Exel6151 . Bang-sensitive *sda* and *para*^{bss1} flies were crossed to either *w*^{1118} or Exel6151 and the progeny tested for seizure penetrance. $p<0.05$. $N\geq5$ for each genotype.

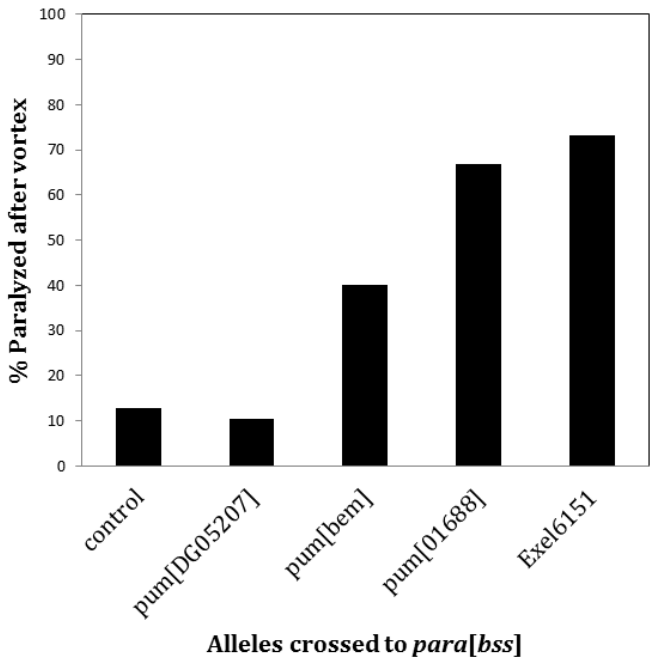
rabbit 488 (Invitrogen), donkey anti-mouse Cy3 highly adsorbed (Jackson Immunoresearch). All concentrations for secondary antibodies were 1:1000.

Adult Immunohistochemistry

Adult brains were prepared as follows: Anaesthetized adults were briefly dipped into ethanol and pinned in sylgard dishes. The cuticle surrounding the head and thorax was removed and the brain and ventral nerve cord were moved to a 4% paraformaldehyde solution for 20 minutes. The brains were briefly washed in PBS and then quickly rinsed three times and 15 minutes three times in PBST. Brains were then blocked in PBST + 10% donkey serum for one hour and placed in primary antibody overnight at 4°C. The brains were given three quick and three 15 minute washes in PBST and placed in secondary antibody overnight at 4°C. The brains were washed overnight in wash solution, after which they were mounted on glass slides in Vectashield (Vector Labs) with hole punch reinforcement sticker used as a spacer between the slide and cover slip, and then stored in the dark at 4°C.

The primary antibodies used are as follows: anti-pumilio B (rat, 1:1000, gift of Paul Macdonald), anti elav (mouse, 1:200, Developmental Studies Hybridoma Bank), anti-bruchpilot (mouse, 1:100, Developmental Studies Hybridoma Bank), anti-GFP (rabbit, 1:2000, Invitrogen), and anti-βgal (mouse, 1:200, Promega). Secondary antibodies are as follows: goat anti-rat 488 (Invitrogen), goat anti-rabbit 488 (Invitrogen), donkey anti-mouse Cy3 (#715-165-151, Jackson Immunoresearch), goat anti-mouse Alexa 594 (Invitrogen). All concentrations for secondary antibodies were 1:1000.

A



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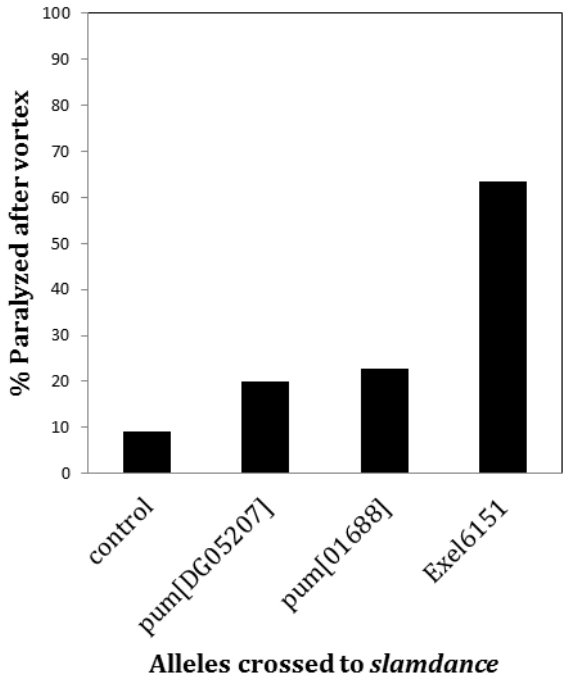

Figure 3.3 Alleles of the gene *pumilio* enhance the penetrance of bang-sensitivity of (A) $para^{bss1}$ and (B) *slamdance* heterozygotes compared to $para^{bss1}$/+ and *sda*/+, respectively heterozygotes and tested by vortexing. N≥40 for each allele.

Electrophysiology

Electrophysiology was based on *Drosophila* seizure assays for epilepsy mutants [22]. Adult flies 2-5 days post eclosion were sorted into vials the day prior to recording and allowed to recover. Flies were individually handled without CO_2 anesthesia using a modified Tetra Whisper 100 aquarium pump and mounted on dental wax on a glass slide, leaving the dorsal surface of the fly exposed. Two electrodes were inserted into the head underneath the antennae, a recording electrode inserted into the thorax, and the neutral electrode into the abdomen. The stimulating electrode was connected to the Grass S48 Stimulator (Grass Instruments), and the recording electrodes to the differential AC amplifier (Model 1700, A-M Systems) to the oscilloscope (TDS1002B, Tektronix). All electrodes were made of tungsten and the ends stripped of insulation. To induce seizure behavior, stimuli of varying voltage were given to the flies (300 ms train at 200 pps, each pulse being 0.5 ms). Recordings were acquired through Digidata 1322A (Axon Instruments) and recorded using Clampex 8.2 (Axon Instruments).

Imaging

All confocal imaging was done using on the Leica SP2 at the Cornell Imaging Facility. Images were taken using either the 40x oil immersion lens and 20x or 63x water immersion lens at 1x or 2x zoom. All images were cropped using Adobe Photoshop CS4 or Adobe Photoshop Elements 10 and figures were arranged using Adobe Illustrator CS4.

Statistics

All electrophysiology data is shown using mean ± standard error of the mean (SEM). Significance is considered at $p<0.05$ and is determined using the unpaired t-test.

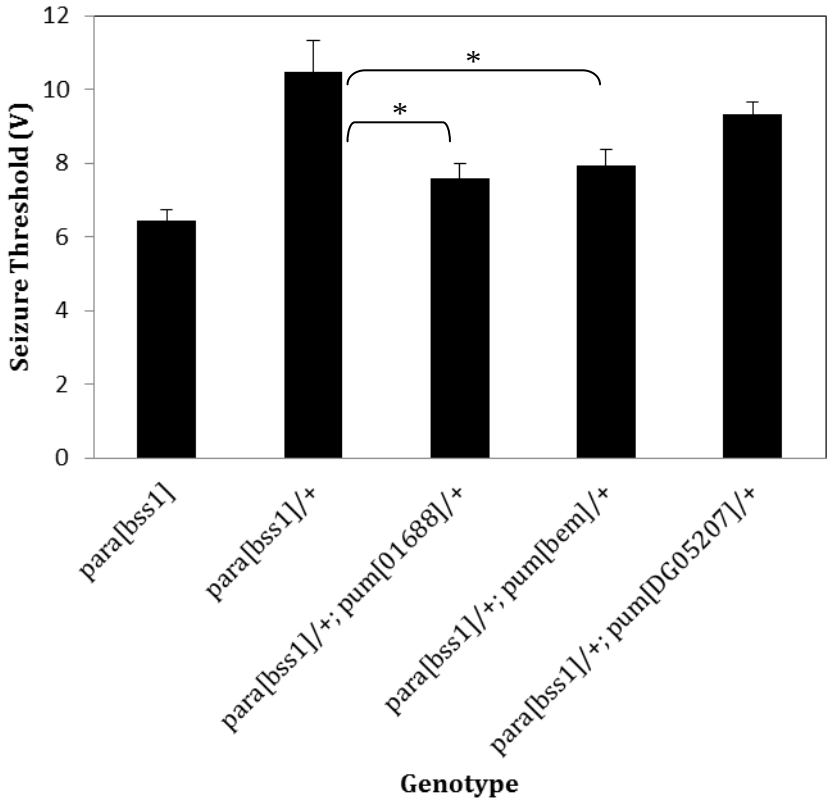

Figure 3.4 Seizure threshold of *para*^{bss1} heterozygotes are reduced by alleles of *pum.* *para*^{bss1} flies were crossed to various *pum* alleles and tested for seizure after vortexing. The threshold *para*^{bss1}/+; *pum*^{01688}/+ is significantly lower than that of *para*^{bss1}/+, $p<0.01$. *para*^{bss1}/+; *pum*^{bem}/+ seizure threshold is also significantly lower than control, $p<0.05$. N>9 for each category except *pum*^{DG0527}, where N=3.

3.4 Results

3.4.1 Mutations in pumilio enhance penetrance and threshold of seizure behavior in a bang-sensitive background

In a previous mutant screen, we searched for deficiencies that increased the occurrence of seizures in a sensitized seizure-susceptible background. This screen was performed in the *slamdance* (*sda*) background, which is a member of the group of *Drosophila* seizure mutants known as bang-sensitives, due to the phenotype of displaying strong seizures after being given a mechanical or electrical stimulus. One of the deficiencies found in this screen with the strongest effects on increasing seizure penetrance in *sda* heterozygotes was the Exelixis deficiency *Exel6151*. Further behavior testing showed that this deficiency enhanced bang-sensitivity in two other bang sensitive lines, *paralytic*^{bangsenseless1} (*para*^{bss1}) and *easily shocked* (*eas*) (Figure 3.1). To confirm that this enhancement in bang-sensitivity has a neuronal basis, we used electrophysiology to test threshold of the voltage stimulus given to the fly brain to induce a seizure. In both the *para*^{bss1} and *sda* backgrounds, the presence of *Exel6151* significantly reduced the seizure threshold compared to *para*^{bss1}/+ and *sda*/+ controls ($p<0.05$) (Figure 3.2).

Since the deficiencies within the Exelixis collection are well characterized and have defined breakpoints, we knew that there are four genes deleted within *Exel6151*, three of which are moderately to highly expressed in neural tissue. In the screen, we targeted genes that are highly expressed in the nervous system since these would be most likely to have a direct effect on enhancing seizure susceptibility. Therefore, we obtained transposon insertions for the three genes and crossed these to *sda*. The progeny, which were heterozygous for both the transposon and *sda*, were tested for bang-sensitivity. The progeny tested are heterozygous for both the transposon and *sda*. Of the three genes tested, the gene *pumilio* (*pum*) was the most effective at enhancing bang-sensitivity, though the other two genes gave slight enhancement as well (Table B.10). We tested

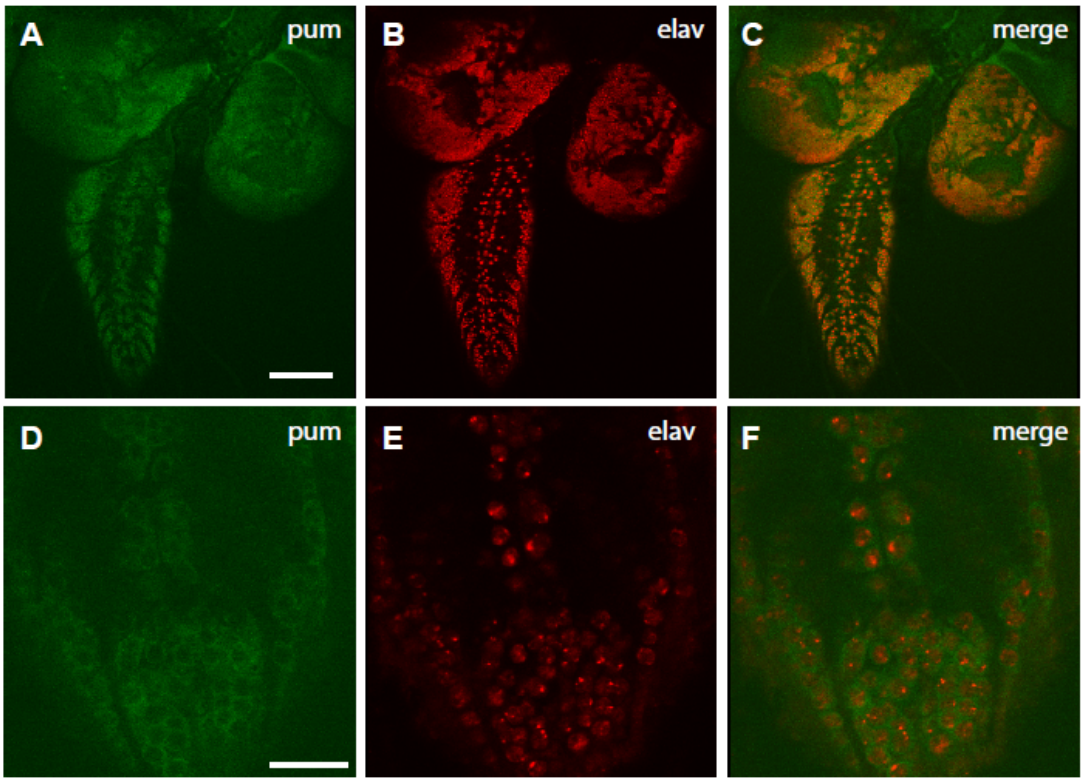

Figure 3.5 Expression of pumilio in third instar larvae brains. Confocal images of pumilio (green) and elav (red) show pumilio expression in most neurons. Images of the whole brain are shown (A-C) and focused on the posterior end of the ventral ganglion (D-F). Scale bar, 100 μm (A-C) and 10 μm (D-E).

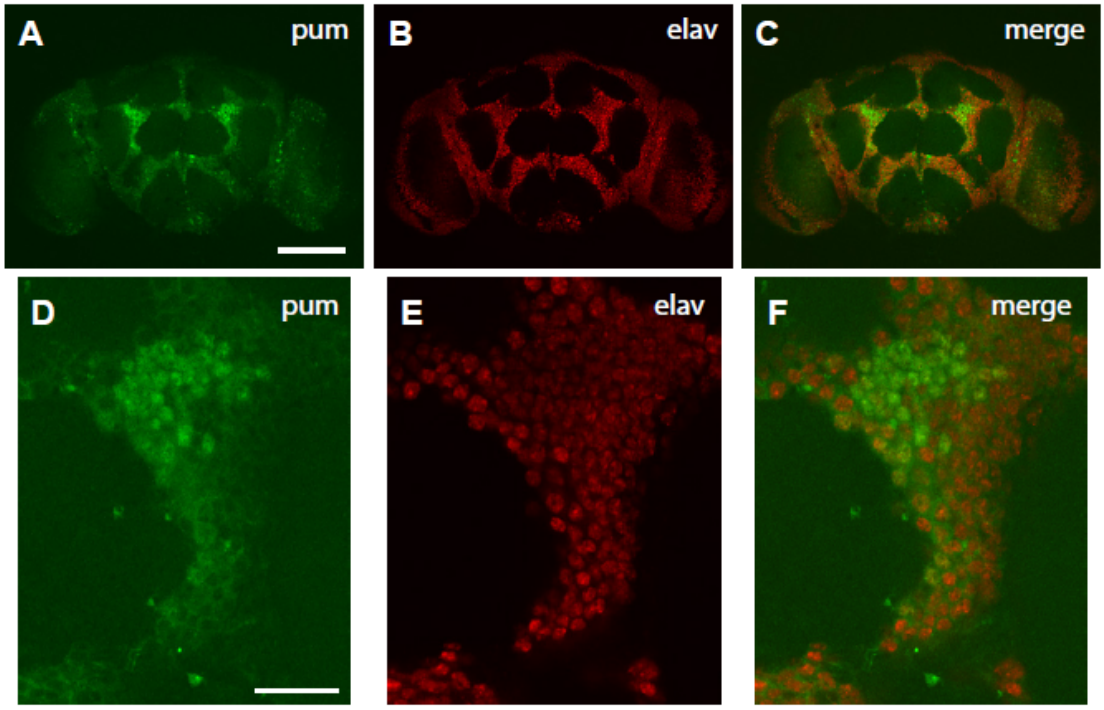

Figure 3.6 Expression of pumilio in adult brains. Confocal images of pumilio (green) and elav (red) show pumilio expression in most neurons in the whole brain (A-C) and a higher magnification of the central brain area (D-F). Scale bar, 100 μm (A-C) and 10 μm (D-E).

other alleles of *pum* and found that seizure penetrance was enhanced in both the *sda* and $para^{bss1}$ background by several alleles, though most strongly by pum^{01688} (Figure 3.3). Electrophysiology tests of these various *pum* alleles showed that there is a significant decrease in seizure threshold in $para^{bss1}$/+; pum^{01688} compared to controls ($p<0.05$), and while the threshold of lines that use other *pum* alleles are decreased compared to control, the difference was not found to be significant (Figure 3.4).

3.4.2 Pumilio is expressed in larval and adult neurons

We decided to investigate the expression pattern of Pum protein in fly larvae and adults since most immunohistochemistry for Pum has previously been done mainly in embryos and adult reproductive organs [23], but not in many other structures. *pum* is well known to be involved in processes such as synaptic transmission, dendrite morphogenesis, and other neurobiological functions [15], [19], so we examined Pum expression in the nervous system. We double stained third instar larvae for Pum [23] and elav, a neuronal marker, and observed that all cells that are positive for cytoplasmic staining of Pum also co-stained with elav in their nuclei (Figure 3.5), indicating that Pum staining is exclusively neuronal, supporting previous work done by Menon *et al* [24]. Similarly, we stained adult brains with pum and elav antibodies and observed that pum staining occurred only in neuron cell bodies (Fig 3.6). We also double stained with Pum and Bruchpilot (Brp), a synaptic marker, to show that pum is located in the cell bodies and not in the neuropil (Figure C.1). Double staining with a glial marker, repo, verified that pum expression is limited to neurons as it does not overlap with repo (Figure C.2).

3.4.3 Targeted knockdown of pum in neurons enhances seizure susceptibility

Since we observed that Pum is expressed in neurons, we hypothesized that targeted knockdown of Pum in neurons or subsets of neurons using RNAi could produce a similar increase in seizure penetrance as in our previous tests with

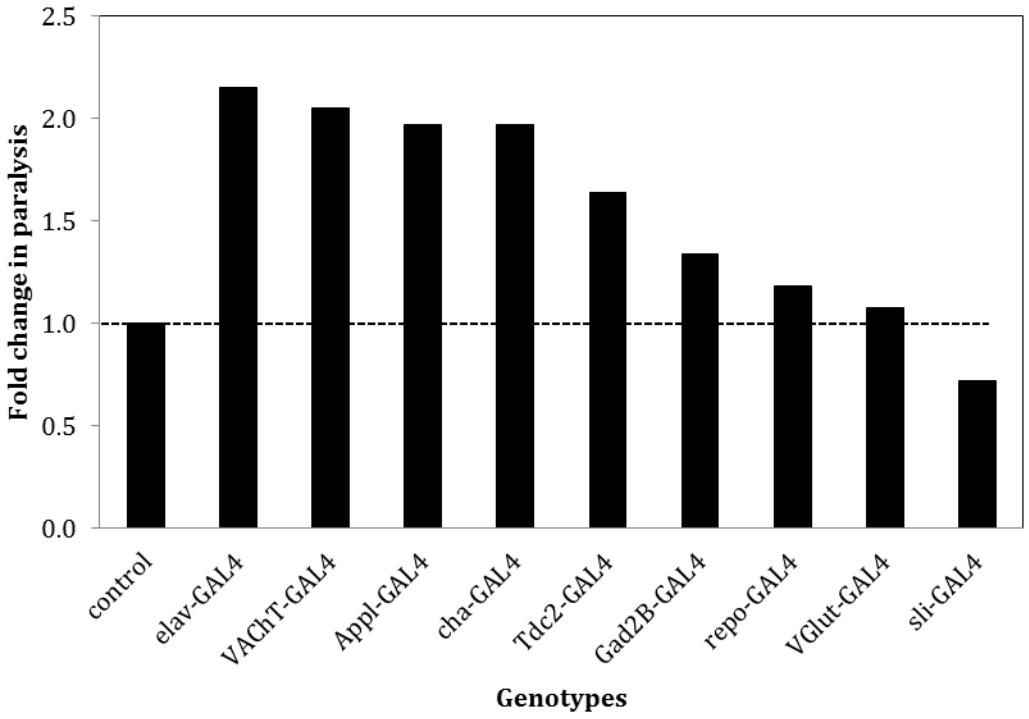

Figure 3.7 Changes in seizure penetrance due to targeted knockdown of *pum* in a *para*^{bss1}/+ background. The graph represents the fold change in paralysis of flies after vortexing compared to control (*para*^{bss1}/+). Pumilio is knocked down by RNAi using specific GAL4 drivers in a *sda* background. Elav = panneural, VAChT = cholinergic neuron, cha = cholinergic neurons, appl = panneural, tdc = octopaminergic neurons, gad1 = GABA-ergic neurons, repo = glia, vglut = glutamatergic neurons, slit = glia. N≥20 for each genotype.

pum transposon alleles and deletions. We tested this hypothesis in two bang-sensitive backgrounds by crossing either *para*^{bss1}; *pum*-RNAi or *pum*-RNAi; *sda* to various GAL4 lines that target the nervous system. The candidate GAL4 lines targeted cholinergic neurons, *Choline acetyltransferase* (*Cha)* and *Vesicular acetylcholine transporter* (*VAChT*), octopaminergic neurons, *tyrosine decarboxylase* (*tdc*), GABAergic neurons, *Glutamic acid decarboxylase* (*Gad1*), glutamatergic neurons, *Vesicular glutamate transporter* (*VGlut*), all neurons, *elav* and *β amyloid protein precursor-like* (*Appl*), and glia, *reversed polarity* (*repo*) and *slit* (*sli*).

We used antibody staining to check the effectiveness of Pum knockdown by RNAi by comparing control *pum*-RNAi/+ flies to *elav-GAL4*/+; *pum*-RNAi/+ after double staining with Pum and elav antibodies. In third instar larvae, the Pum labeling in the ventral ganglion is reduced (Figure 3.10) as well as throughout the rest of the larval brain (Figure C.4). The results are similar in the adult brain, where knockdown of Pum results in an almost complete lack of Pum staining in the brain and thoracic abdominal ganglion (Figure 3.11). This reduction of Pum staining is apparently only when we use elav-GAL4 as the driver, since when we knocked down Pum in smaller subsets of neurons such as motor neurons, glutamatergic neurons, cholinergic neurons, and GABA-ergic neurons, we did not observe a change in the staining pattern of Pum (Figure C.3). However, this could be an effect of the driver strength at driving RNAi and a stronger GAL4 line may cause a clearer difference in Pum pattern.

All of the GAL4 lines listed above were crossed to *para*^{bss1}; *pum*-RNAi and tested for seizure behavior, and we found that panneural drivers (*elav* and *Appl*) as well as cholinergic neuron drivers (*Cha* and *VAChT*) caused a twofold or higher increase in bang-sensitivity in the tested adults (Figure 3.7, Table C.1). We expected that since *pum* alleles are able to enhance seizure susceptibility in multiple lines of bang-sensitive mutants, RNAi knockdown of *pum* should be

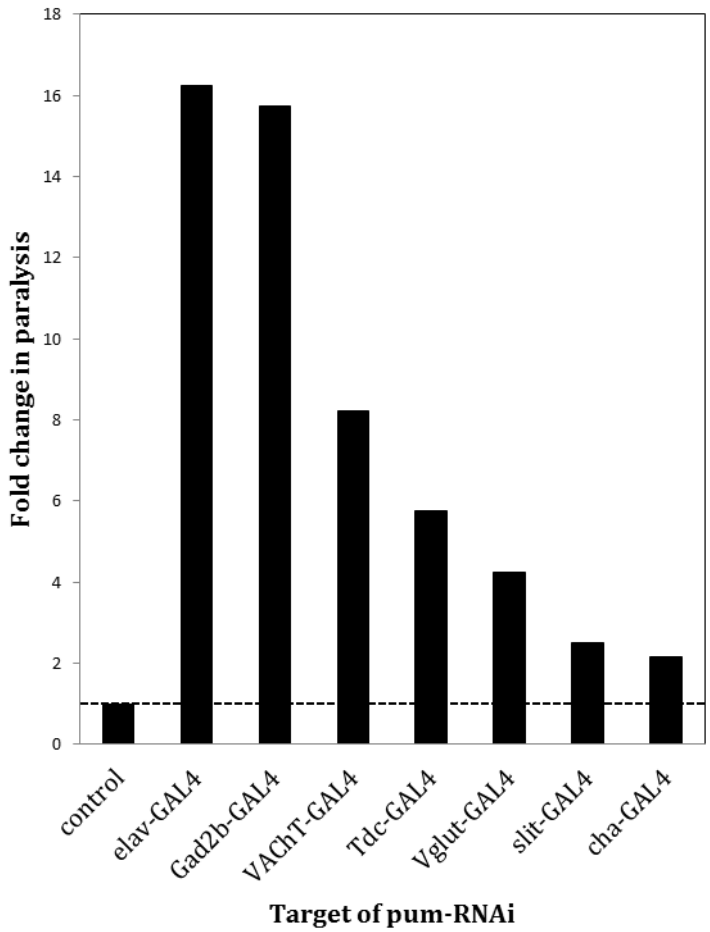

Figure 3.8 Effects of *pum* knockdown by *elav*-GAL4 in a *sda*/+ background. The graph represents fold change in flies showing paralysis after vortexing compared to control (*sda*/+) . There is an increase in paralysis when pum is knocked down in GABA-ergic neurons by Gad2B-GAL4, and to a lesser extent in cholinergic neurons *VAChT*-GAL4 and a larger increase in paralysis when pum is knocked down by a panneural driver, *elav*-GAL4. N≥65 for each genotype.

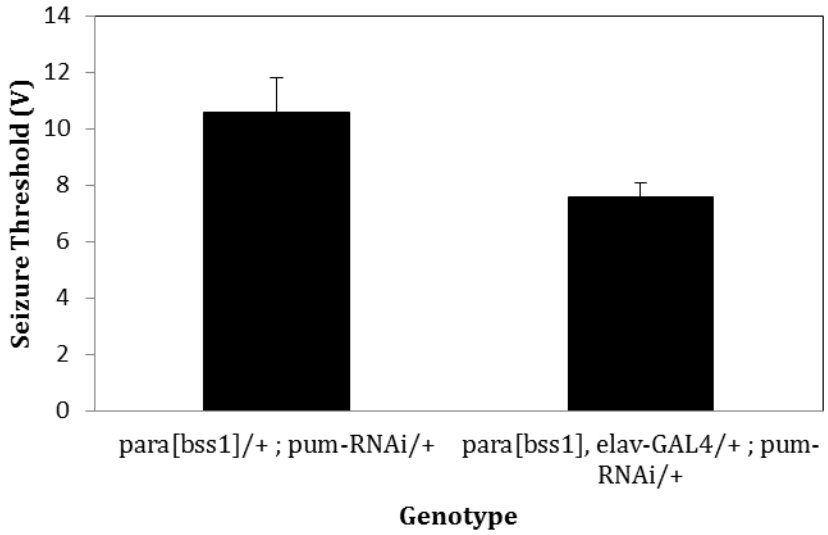

Figure 3.9 Seizure threshold is lower in adults with *pum* knockdown in neurons. *para*^{bss1}; *pum-RNAi* flies were crossed to panneural driver *elav*-GAL4 and the seizure threshold for each genotype was determined. Knockdown of pum in neurons significantly lowered the seizure threshold compared to control. $p<0.05$, $N\geq9$ flies for both genotypes.

effective in enhancing seizure penetrance in other BS mutants. When we repeated this experiment by crossing *pum*-RNAi; *sda* to the different drivers, we found that cholinergic, similar to the previous experiment, and also GABA-ergic neuron GAL4 lines were also able to increase penetrance of seizure (Figure 3.8, Table C.2).

To confirm that the increase in bang-sensitivity penetrance was due to an increase in neuronal excitability, we measured the seizure threshold of the $para^{bss1}$, *elav*-GAL4/+; *pum*-RNAi/+ flies, which had given the strongest enhancement phenotype in the vortex testing. We found that the seizure threshold for this genotype is significantly lower in $para^{bss1}$, *elav*-GAL4/+; *pum*-RNAi/+ flies (7.56±0.53V) compared to the control, $para^{bss1}$/+; *pum*-RNAi/+ (10.58±1.22V), $p<0.05$ (Figure 3.9, Table C.3). These recordings, along with the behavioral testing, confirms that knockdown of Pum in neurons does enhance the penetrance of seizure behavior. Specific knockdown in cholinergic neurons also produces a behavioral effect, though we have not measured seizure thresholds in these flies.

From behavior testing, we observed that targeting Pum knockdown through the cholinergic neuron driver VAChT-GAL4 was also effective at increasing the penetrance of bang-sensitivity in the $para^{bss1}$ and *sda* backgrounds (Figure 3.8, Figure 3.9). One possibility for this phenotype is that cholinergic neurons comprise a large subset of the cells stained by Pum, so we used antibody staining to determine the pattern of VAChT-positive cells compared to Pum-positive cells. We constructed an *nls*-GFP; *VAChT*-GAL4 stock to express GFP in the nuclei of cells that express VAChT and performed double antibody staining against Pum and GFP in these flies. In third instar larvae, we observed in the ventral ganglion, many VAChT-expressing nuclei are found within cells with Pum cytoplasmic staining (Figure 3.12 D-F) and a smaller number of VAChT-expressing nuclei are found in the brain lobes (Figure 3.12 A-C). In adults, we also observed that there

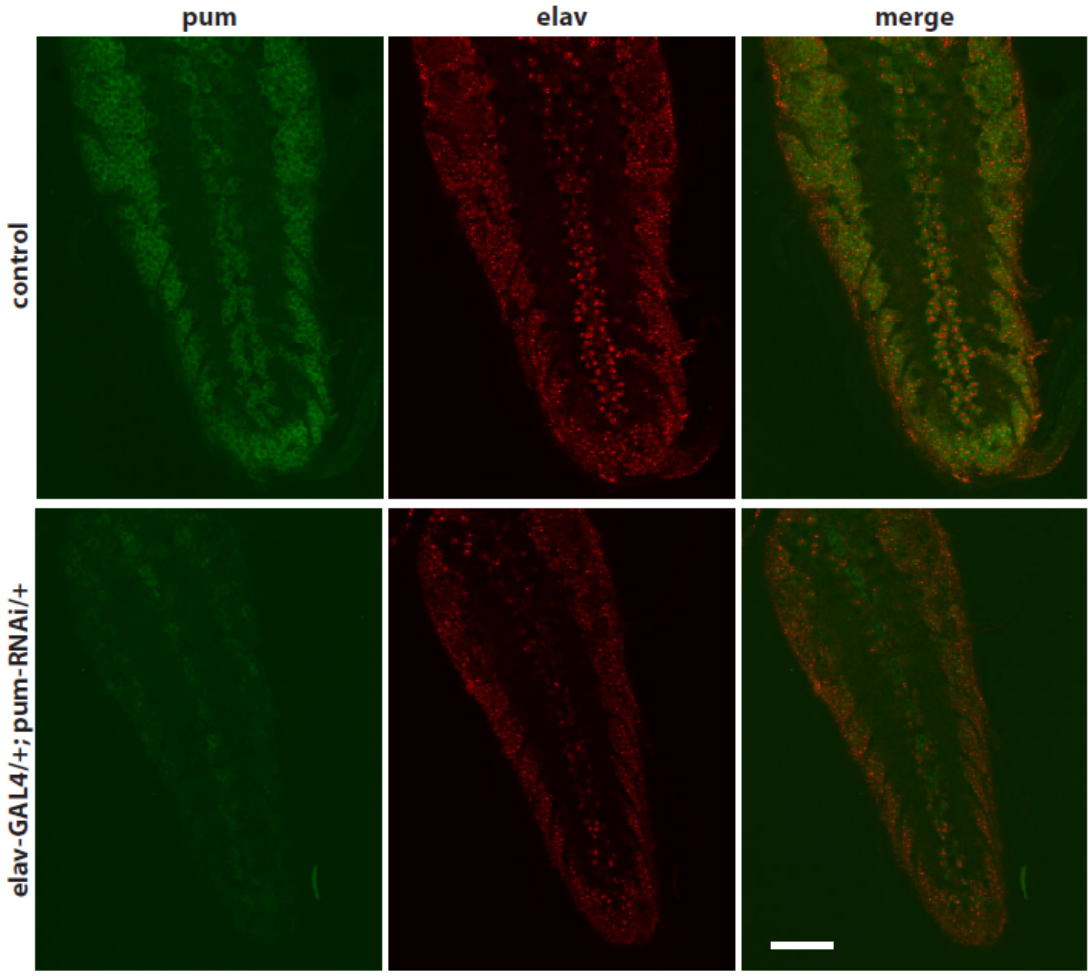

Figure 3.10 Knockdown of *pum* in neurons of third instar larvae. Knockdown of pumilio in neurons using RNAi and *elav*-GAL4 driver. Confocal images of third instar larvae brains, specifically ventral ganglion, stained with pumilio antibody and elav antibody. Scale bar, 50 μm.

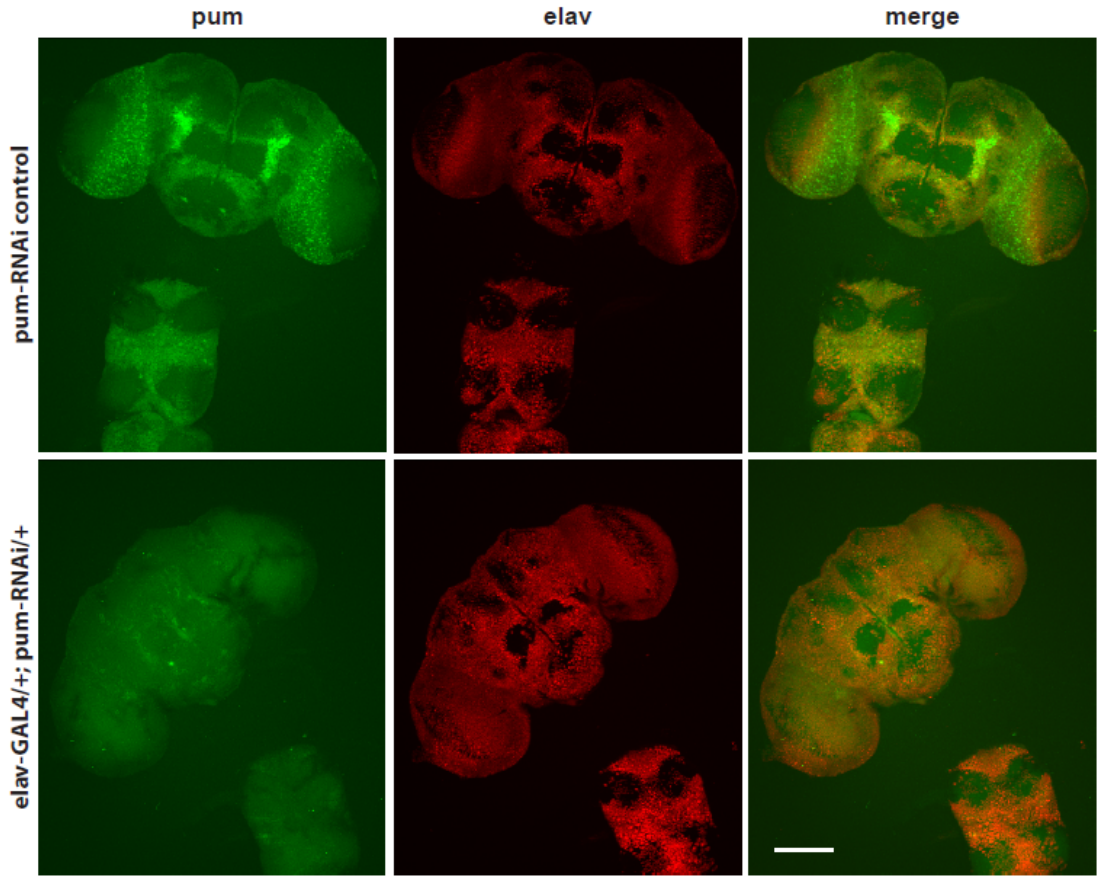

Figure 3.11 Knockdown of *pum* in neurons of adult brains. Maximum projection of confocal z-series of adult brains stained with pumilio and elav. Scale bar, 50 μm.

are many nuclei expressing VAChT in cells that also have Pum staining (Figure 3.13), suggesting that cholinergic neurons comprise a significant fraction of Pum-positive neurons.

3.4.4 Disruption of pumilio expression during the entire life cycle causes enhancement of neuronal excitability

Thus far all of our tests have used alleles of *pum* or RNAi knockdown of Pum throughout the entire life cycle of the fly. Could Pum knockdown in specific stage of the life cycle cause enhancement of bang-sensitivity? To test this, we used a GAL80ts line recombined with *pum*-RNAi to perform knockdown of Pum during specific stages of the fly life cycle. We crossed this line to *elav-GAL4*, *sda* and raised the progeny at the restrictive temperature during specific periods of the life cycle. GAL4 is active only at the restrictive temperature, so knockdown of Pum occurs only during this time. In this initial test, all eggs were laid at 25°C. One set of flies was placed at 29°C during all larval stages, another set was placed at 29°C from pupation to adulthood, and another set of flies were raised at 29°C from the first instar stage and onwards. We collected the flies and tested them for bang-sensitivity, and observed that only flies raised for their entire life cycle at the restrictive temperature showed a large increase in seizure penetrance (Figure 3.14). Those raised at the restrictive temperature through pupation and eclosion showed a slight increase in bang-sensitivity. Pum expression during pupation is important to normal development, as overexpression of Pum during this period can cause lethality (Table C.4). Repeating this test with smaller time windows may help to refine the critical period suggested by these results.

2.5 Discussion

In our study, we have shown that Pum is expressed in neurons in the larval and adult nervous system. Specifically, Pum is found in most neurons as shown by its overlap with elav, a well-known neural marker, and knockdown of Pum using

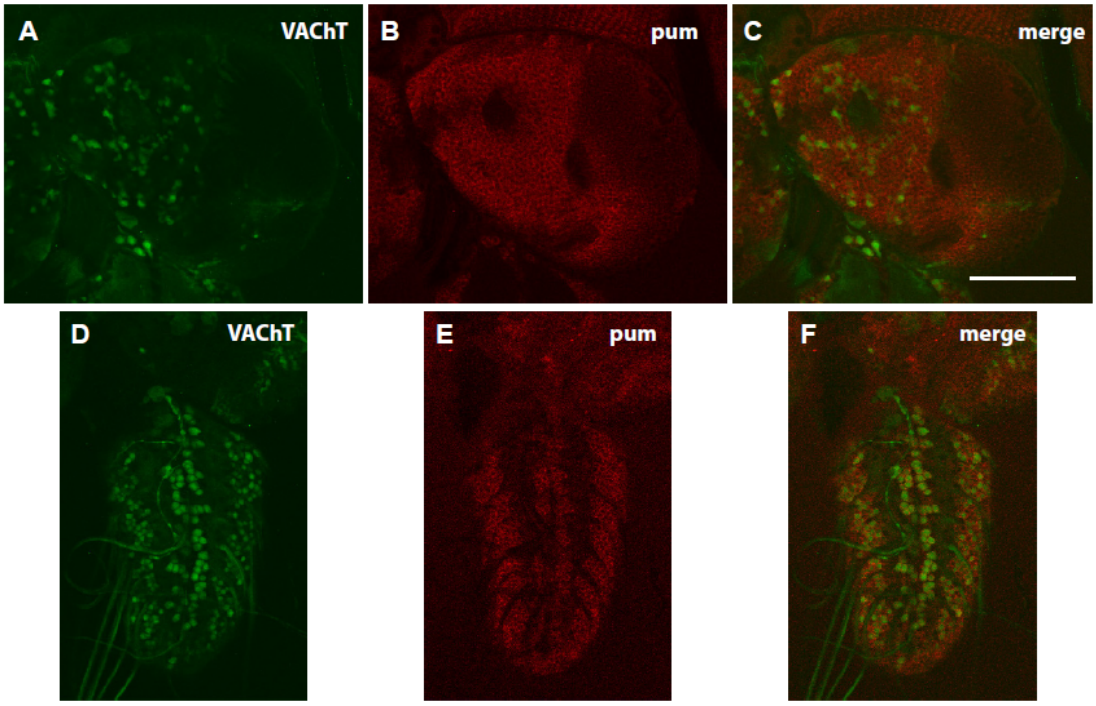

Figure 3.12 Localization of Pum in cholinergic neurons in third instar larval brains. Anti-GFP and anti-Pum staining with VAChT-GAL4 driving a nuclear localized GFP in the larval brain, specifically the lobe (A-C) and the ventral ganglion (D-F). Scale bar is 100 μm.

RNAi using a panneural driver was able to abolish much of the Pum staining in both larval and adult brains. Knockdown of Pum using drivers that targeted subsets of neurons did not noticeably remove Pum staining. One explanation for this is that different GAL4 drivers have different levels of effectiveness. For example, we used two different *elav*-GAL4 lines (C155 and 3A4) in our experiments, and C155 had a much stronger effect on expression and behavior than 3A4. It is possible that the GAL4 lines we used to target neuronal subsets were not able to drive *pum*-RNAi effectively.

Earlier studies in adult Pum expression indicated that the protein was expressed throughout the central nervous system, especially in the Kenyon cells of the mushroom bodies [18]. We did observe increased staining ventral to the mushroom bodies, between the antennal lobes and mushroom bodies. However, the Kenyon cells are located more dorsal to the mushroom bodies [25] and are unlikely to be the area with increased Pum staining in our figures. One unusual aspect of the cells we stained is that while Pum staining is very high, elav staining in these cells is also lower than that of surrounding cells. As elav is not expressed in neuroblasts or earlier stages, but does appear in all neurons, a possible explanation may be that these cells are in the process of differentiating into neurons. While cell division and differentiation in the adult brain is rare, recent work has shown that there is indeed cell proliferation in the adult fly brain. These cells are also located around the area of the antennal lobe [26].

From a deficiency screen, we identified *pum* as an enhancer of bang-sensitive behavior. The deficiency that deleted *pum* was able to enhance the seizure penetrance in three different bang-sensitive mutants, $para^{bss1}$, *sda*, and *eas* that are known to have different seizure thresholds but cause very similar seizure-paralysis-seizure phenotypes [27]. $para^{bss1}$ is a gain-of-function mutation in a voltage-gated Na^+ channel that causes a change in Na^+ inactivation [28]. *sda* encodes a mutation in an aminopeptidase that increases persistent Na^+ current

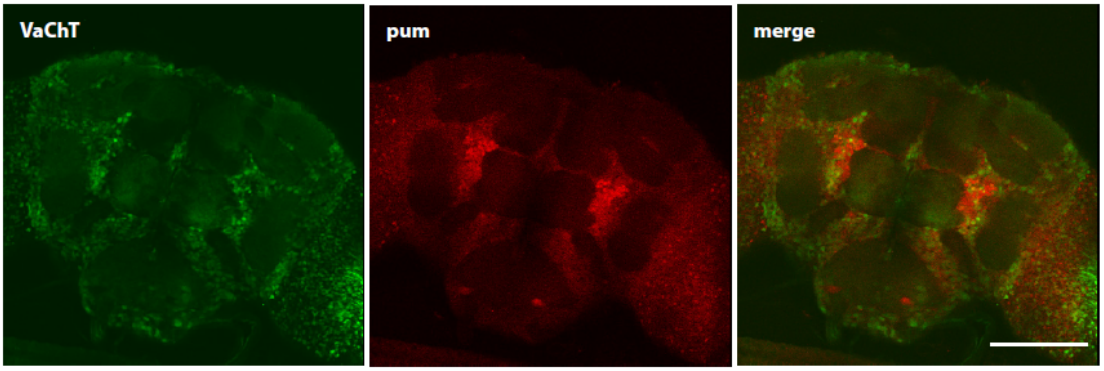

Figure 3.13 Localization of Pum in cholinergic neurons in adult brains. Anti-GFP and anti-Pum double labeling of VaCHT-GAL4 driving nuclear localized GFP. Scale bar is 100 μm.

[29]. The mutation in *eas*, which encodes an ethanolamine kinase, is believed to alter membrane phospholipid composition that causes an excitability defect [30]. Because the three different bang-sensitive mutations are very different and cause seizures through different methods, the enhancement of seizure penetrance caused by *pum* is most likely due to a general increase in neuronal excitability instead of being specific to a defect caused by one bang-sensitive mutant. Flies that are mutant for *pum* do not have seizures themselves. However, recent work in mice has shown that those carrying a specific allele of *pum2*, the mouse homolog of *Drosophila pum,* show spontaneous seizures due to handling and have lower seizure thresholds in response to convulsants [31]. This suggests that there may be a closer link between *pum* and seizure behavior than previously thought.

Although Pum is expressed in most and perhaps all neurons, it is possible that the enhancement of seizure susceptibility is due to lack of Pum in a specific subset of neurons. Studies using pum^{bem}, an allele of *pum*, showed that Pum is able to regulate Na^+ current in specific motoneurons, and the pum^{bem} allele is able to increase Na^+ current which would lead to greater neuronal excitability [21]. Therefore, it had been believed that the primary cause of increased neuronal excitability due to *pum* mutations is due to defects in motor neurons. However, we found that knockdown of Pum in glutamatergic neurons, which releases glutamate at the neuromuscular junction (NMJ), in a $para^{bss1}$ or *sda* background did not enhance bang-sensitivity as much as other types of neurons. Instead, the neurons that produced the greatest effect on BS behavior were the cholinergic neurons in the case of the $para^{bss1}$/+ background. A previous study on the vesicular acetylcholinergic transporter (VAChT) showed the defects in VAChT caused defects in the giant fiber pathway, most likely due to reduction in acetylcholine levels at the giant fiber synapses [32]. Studies in mice show that VAChT knockouts have spontaneous release of neurotransmitters at the neuromuscular junction and also have defective NMJ development [33]. It is

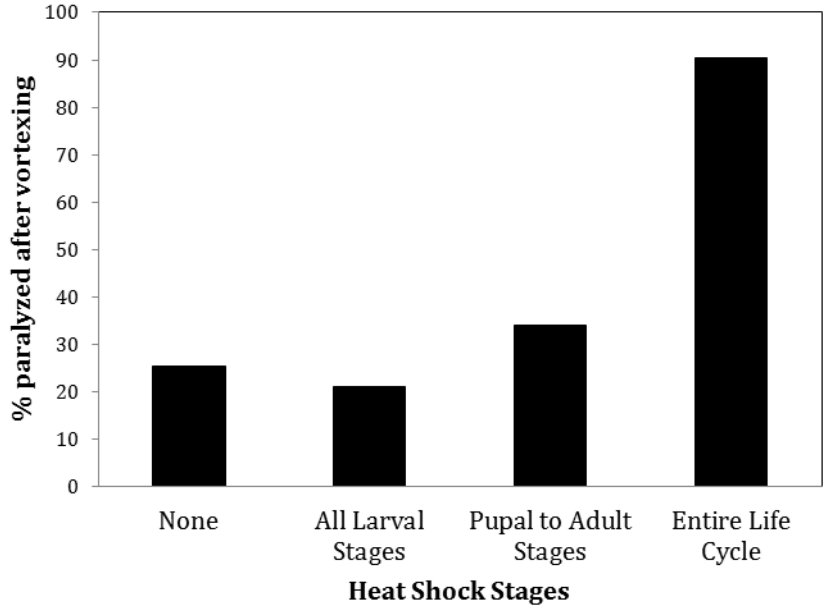

Figure 3.14 Effects of knockdown of pumilio at various stages of fly life cycle on bang-sensitivity penetrance. Knockdown of pum was done by crossing GAL80ts, pum-RNAi flies to *elav*-GAL4, *sda* and raising the *elav*-GAL4/+; GAL80ts, pum-RNAi/+; *sda*/+ progeny at 29°C during the indicated developmental stages. Once the flies reached adulthood, they were tested for bang-sensitivity. N≥20 for each category.

possible that *pum* has a role in normal development of the NMJ in cholinergic neurons, and that lack of Pum leads to defects that cause increased neuronal excitability.

In the *sda*/+ background, while knockdown of Pum in cholinergic neurons does enhance the BS behavior, the greatest change is caused by knockdown of Pum in GABAergic neurons. There is evidence that reduced GABAergic synaptic transmission and the resulting lack of inhibition can lead to seizures, which has been shown both in human epilepsy caused by mutations in genes encoding the $GABA_A$ receptor subunits, GABRA1 and GABRG2, and in the *Drosophila kazachoc* (*kcc*) mutant [34], [35]. Analysis of the *kcc* mutant suggested that influence of altered GABAergic signaling occurs in the mushroom bodies and takes place during the late pupal-early adult period [35]. Interestingly, our results show that normal Pum expression during the late pupal-early adult period is also important for survival, as overexpression of Pum during this stage lowers the survival rate to adulthood. Since the effect of GABAergic neuron-targeted Pum knockdown in seizure behavior is limited to *sda*, we hope to explore the role of *sda* in neural development during these pupal stages.

We have shown in this study that mutations of *pum* in flies cause a strong enhancement in BS seizures. Since Pum is expressed in neurons, knockdown of Pum using neural drivers also replicates this effect, though most potently by panneural drivers, and almost as well by cholinergic, and GABAergic neuron drivers. Conditional knockdown of Pum during specific stages of development showed that a reduction of Pum must occur both in larval and through pupal stages in order for the seizure enhancement to occur. Also, there is a period between third instar and pupal stages that is important for development. With this new knowledge about *pum* and continued refinement of experiments described in this study, we will be able to narrow down the critical location and time window of Pum involvement in neuronal excitability.

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CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

4.1 Brief overview

In the previous chapters, I have discussed a behavioral genetic screen in which we looked for genes that may help our understanding of neuronal excitability, and by extension, the underlying mechanisms and genetics behind epileptogenesis. Following this, I discussed our analysis of one of the genes, *pumilio* (*pum*) that was found in this screen.

4.2 Deficiency screening for enhancers of the bang-sensitive mutant slamdance

Since research in the genetic basis of epilepsy is moving towards large scale or genome wide studies to identify genes associated with epilepsy [1], we hoped to use the tools available to *Drosophila* to efficiently screen for such genes in fly seizure mutants. The goal of this project was to find new genes, previously not implicated as being involved in seizure behaviors or neuronal excitability. We performed an enhancer screen using the seizure phenotype of the bang-sensitive mutant *slamdance.*

4.2.1 Eleven candidate enhancers of bang-sensitivity

We screened chromosome 3R for enhancers of seizure susceptibility using an Exelixis deficiency kit. Out of the 15 Exelixis deficiencies that enhanced the bang-sensitivity of *sda* heterozygotes we discovered 11 candidate genes with known functions. Three of the eleven genes are known to be involved in nervous system development: *Tetraspanin 86D* (*Tsp86D*), CG31140, and *48 related 2* (*fer2*). Two have functions in the secretory pathway: *yata* and *Sorting nexin 3* (*Snx3*). Two encode O_2 and NO sensors. *Guanylyl cyclase β* encodes an NO sensor and *Similar* (*Sima*) is an oxygen sensor. Three genes encode proteins involved in

translational control: *Akt*, *pum*, and CG18616. Only two of eleven genes, *pum* and CG9467, had previously been associated with enhancing neuronal excitability or having homology to epilepsy genes.

4.2.2 Searching for interactors of sda

Ten out of the eleven candidate enhancer genes increased BS seizure penetrance in both the heterozygous $para^{bss1}$ and *sda* background. This was shown indirectly by testing the Exelixis deficiencies that contained these genes and showing the general enhancement to both BS mutants. One of the genes causes seizure enhancement specific to *sda* and may encode a product that is an interactor of only *sda.* Two deficiencies tested in this screen caused synthetic lethality with *sda* but not other bang-sensitive lines. Though we have not yet determined the genes underlying the synthetic lethality, we hope to use these deficiencies to find possible interactors of *sda*.

4.3 pumilio is required during several development stages for normal neuronal excitability

We explored the role of *pum* in enhancing seizure susceptibility after it was discovered in the deficiency screen. Though other work had shown that *pum* was involved in neuronal excitability and growth of the neuromuscular junction [2], [3], they did not show an actual change in behavior. We showed in chapter 3 that mutations in *pum*, or knockdown of Pum by RNAi, can enhance the duration or severity of seizure behavior.

4.3.1 Pumilio is expressed in neurons in larval and adult central nervous systems

We were able to show by using Pum and elav antibody staining that Pum is expressed in most if not all neurons. We also showed that Pum expression is predominantly in neurons, as knocking down Pum using RNAi targeted to neurons abolishes most Pum expression. This allowed us to perform further

studies using *elav*-GAL4 to study the effects of conditional Pum knockdowns at specific points in the life cycle.

4.3.2 Decreasing levels of Pum through mutant alleles or RNAi knockdown enhances bang-sensitive behavior

Mutations in *pum* can cause enhancement of BS seizure penetrance in both the *sda* and $para^{bss1}$ backgrounds. Knockdown of Pum using RNAi targeted to neurons in a BS background also caused a higher penetrance of the BS phenotype. We confirmed these findings using electrophysiology, showing that when Pum is reduced either by mutation or RNAi, the voltage of stimulus required to cause a seizure response is significantly lower than in control flies. These results provided behavioral evidence that *pum* is involved in seizure susceptibility and neuronal excitability. Since targeting neurons caused the enhancement, it is likely that the role of Pum in enhancing BS seizures is due to changes in neural development and not due to early developmental roles in germ cell maintenance and axis patterning.

4.3.3 Pum reduction in GABAergic or cholinergic neurons is effective in enhancing bang-sensitivity

We tested Pum knockdown in various subsets of neurons to determine if Pum function in neuronal excitability is limited to specific neurons. We discovered that in both $para^{bss1}$ and *sda*, knockdown of Pum in cholinergic neurons caused enhancement of BS seizures. In the *sda* background, knockdown of Pum in GABAergic neurons had an even greater effect on seizure behavior. This is the first example showing that Pum expression in specifically cholinergic and GABAergic neurons is needed for normal neuronal excitability.

4.3.4 Enhancement of BS phenotype only occurs when there is Pum knockdown through larval and pupal stages

We initially thought that since seizure behavior is only apparent in adult bang-sensitive mutants and not in larvae, that loss of Pum in the development of the adult brain (during the pupal stage) would be sufficient to cause enhancement in bang-sensitive behavior. However, we found that knockdown of Pum in neurons needs to occur through the majority of the life cycle post hatching in order for the BS seizure enhancement to occur. It is likely that defects in neural development in larval stages are exacerbated by further effects of Pum knockdown during the development of the adult nervous system, leading to a large increase in BS seizure penetrance.

4.4 Future directions

4.4.1 Deficiency screening for enhancers and suppressors of bang-sensitivity

As with most genetic screens, the screen itself is only the very beginning of the project. Our screen and subsequent study of *pum* leaves us with other 9 candidate enhancer genes of neuronal excitability. The next steps would be an analysis of each of these genes similar to what we have done for *pum.* We would need to ascertain where these genes are expressed, what developmental stages are critical for the enhancer phenotype, and how each gene contributes to the seizure phenotype. We will also need to investigate how the genes affect the *sda* mutant that leads to the seizure enhancement.

Overall, our screen only covered a small portion of the *Drosophila* genome. Since the screen was successful for discovering enhancer genes, a more expansive screen using larger sets of different deficiency kits would allow us to discover more enhancer genes and potential interactors of *sda*. If we continue the screen with the restriction of searching for genes with expression in the nervous system, we may miss some genes, but since the *sda* phenotype is caused by altered ionic current in neurons, it is reasonable to restrict our search to genes expressed in the nervous system.

4.4.2 pumilio and its roles in neuronal excitability

We have only begun to narrow down the windows of Pum function in relation to enhancement of bang-sensitivity, both spatially and temporally. While we have found that Pum expression in larval stages and in pupal stages are needed for the enhancement of BS seizures, we do not know if this means that Pum knockdown must take place over this entire period, if there is one time period, or multiple developmental stages. Further testing can help us to figure these critical developmental stages, and this knowledge can subsequently be used to test specific neuronal subsets, such as cholinergic and GABAergic neurons. Since these represent a population of excitatory neurons and a population of inhibitory neurons, respectively, it is possible that Pum functions through more than one mechanism or pathway in order to cause the enhancement of BS behavior.

Pum most likely regulates a large number of genes, as revealed by a screen mRNAs with Pum binding sequences[4], though a screen for synaptic targets of Pum only resulted in a small number of genes [5]. It is possible that some of these synaptic targets of Pum also regulate neuronal excitability, and that behavior testing using the same methods as described in the deficiency screen can be used to determine this. We hypothesize that testing seizure behavior in genes that are targets of Pum may allow us to uncover the process by which Pum is able to enhance BS behavior.

It is our belief that that this work will contribute to the role of *Drosophila* as a model system of epilepsy by expanding the available repertoire and understanding of neuronal excitability mutations.

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APPENDIX A

Table A.1
Preliminary Exelixis deficiency screen results

Deficiency	**Chromosomal Location**	**Effect on *sda***	**# of genes deleted**	**Likely "enhancer"**
Df(3R)Exel6141	82B3-82C4	enhance	10	unknown
Df(3R)Exel6202	96D1-96E2	enhance	33	unknown
Df(3R)Exel6140	82A3-82A5	enhance	11	*complexin*
Df(3R)Exel6152	85C11-85D2	enhance	4	*pumilio*

APPENDIX B

SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Table B.1
Complete list of Bloomington fly stocks used

Bloomington Stock Number	Genotype
458	P{w$^{+mW.hs}$=GawB}elav^{C155}
507	gl^{2} e^{4}
1799	w*; P{w^{+mC}=GAL4-Hsp70.PB}89-2-1
1931	Df(3R)by10, red^{1} e^{1}/TM3, Sb^{1} Ser^{1}
3125	Df(3L)fz-GS1a, P{w^{+tAR} ry$^{+t7.2AR}$=wA^{R}}66E/TM3, Sb^{1}
4381	bw^{1}; Rop^{G27} st^{1}/TM6B, Tb^{+}
4775	w^{1118}; P{w^{+mC}=UAS-GFP.nls}14
4776	w^{1118}; P{w^{+mC}=UAS-GFP.nls}8
5458	w*; pros^{17}/TM6B, Tb^{1}
5905	w^{1118}
6190	svp^{1}/TM3, Sb^{1} Ser^{1}
6326	w^{1118}
6782	w*; P{w^{+mC}=lacW}pum^{bem}/TM6
6793	w*; P{w^{+mC}=Cha-GAL4.7.4}19B P{w^{+mC}=UAS-GFP.S65T}T2
6798	w^{1118}; P{w^{+mC}=Cha-GAL4.7.4}19B/CyO, P{ry$^{+t7.2}$=sevRas1.V12}FK1
6800	w*; P{w^{+mC}=nrv2-GAL4.S}3
7010	w^{1118}; P{w^{+mC}=Ddc-GAL4.L}4.3D
7017	w*; P{w^{+mC}=tubP-GAL80^{ts}}2/TM2
7019	w*; P{w^{+mC}=tubP-GAL80^{ts}}20; TM2/TM6B, Tb^{1}
7108	w*; P{w^{+mC}=tubP-GAL80^{ts}}10; TM2/TM6B, Tb^{1}
7347	y^{1} w^{67c23}; P{y$^{+t7.7}$=Mae-UAS.6.11}nos^{UY4741}
7415	w^{1118}; P{w^{+m*}=GAL4}repo/TM3, Sb^{1}
8760	w*; P{w^{+mC}=GAL4-elav.L}3
8848	w*; P{w^{+mC}=ple-GAL4.F}3
8968	w^{1118}; Df(3R)ED5516, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED5516/TM6C, cu^{1} Sb^{1}
9086	w^{1118}; Df(3R)ED5591, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED5591/TM6C, cu^{1} Sb^{1}
9200	w^{1118}; Df(3R)ED5220, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED5220/TM3, Ser^{1}
9206	w^{1118}; Df(3R)ED5573, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED5573/TM3, Ser^{1}
9227	w^{1118}; Df(3R)ED5428, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED5428/TM6C, cu^{1} Sb^{1}
9313	w*; P{w^{+mC}=Tdc2-GAL4.C}2
9338	w^{1118}; Df(3R)ED5296, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED5296/TM6C, cu^{1} Sb^{1}
9482	w^{1118}; Df(3R)ED10642, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED10642/TM6C, cu^{1} Sb^{1}
9516	Canton-S-iso3A
9580	w*; P{w^{+mC}=GAL4-sli.S}3
9622	w^{1118}; Df(3R)BSC196/TM6B, Tb^{+}
9699	w^{1118}; Df(3R)BSC222/TM6B, Tb^{+}
10344	ry^{506} P{ry$^{+t7.2}$=PZ}sda^{03884}, l(3)03884^{03884}/TM3, ry^{RK} Sb^{1} Ser^{1}
10967	w^{1118}; PBac{w^{+mC}=PB}CG2747^{c02682}/TM6B, Tb^{1}

11544	P{ry$^{+t7.2}$=PZ}pum^{01688} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}
11590	P{ry$^{+t7.2}$=PZ}CtBP^{03463} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}
11604	ry^{506} P{ry$^{+t7.2}$=PZ}repo^{03702}/TM3, ry^{RK} Sb^{1} Ser^{1}
11627	ry^{506} P{ry$^{+t7.2}$=PZ}Akt1^{04226}/TM3, ry^{RK} Sb^{1} Ser^{1}
11720	P{ry$^{+t7.2}$=PZ}eIF-4E^{07238} ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}
12128	y^{1} w*; P{w^{+mC}=lacW}Vha55^{j2E9}/TM3, Sb^{1}
12146	y^{1} w*; P{w^{+mC}=lacW}l(3)j5C7^{j5C7}/TM6B, Tb^{1}
13221	y^{1} w^{67c23}; P{y$^{+mDint2}$ w$^{BR.E.BR}$=SUPor-P}Teh1^{KG02438}
13221	y^{1} w^{67c23}; P{y$^{+mDint2}$ w$^{BR.E.BR}$=SUPor-P}Teh1^{KG02438}
13427	y^{1} w^{67c23}; ry^{506} P{y$^{+mDint2}$ w$^{BR.E.BR}$=SUPor-P}CG31140^{KG01345}
15013	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}CG6439^{EY00276}
15077	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}kay^{EY01644}/TM3, Sb^{1} Ser^{1}
15221	y^{1}; ry^{506} P{y$^{+mDint2}$ w$^{BR.E.BR}$=SUPor-P}Gycbeta100B^{KG09937} stops^{KG09937}/TM3, Sb^{1} Ser^{1}
15965	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}CG18005^{EY06611}
16029	y^{1} w^{1118}; PBac{w^{+mC}=5HPw^{+}}Fas1^{A071}/TM3, Sb^{1} Ser^{1}
16364	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}Glut4EF^{EY03156}
16668	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}CG6359^{EY05688}
16690	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}nonA-l^{EY05999}
17837	w^{1118}; PBac{w^{+mC}=RB}CG6574^{e00293}
18407	w^{1118}; PBac{w^{+mC}=WH}CG7918^{f01078}/TM6B, Tb^{1}
18502	w^{1118}; PBac{w^{+mC}=WH}Ank2^{f02001} CG32373^{f02001}/TM6B, Tb^{1}
19012	w^{1118}; PBac{w^{+mC}=WH}CG10326^{f06814} CG10324^{f06814}
20472	y^{1} w^{67c23}; P{y$^{+t7.7}$ w^{+mC}=wHy}cpx^{DG23508}
20582	y^{1} w^{67c23}; P{y$^{+t7.7}$ w^{+mC}=wHy}Mcm5^{DG12108}
20714	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}CG5903^{EY11938}
21042	y^{1} w^{67c23}; P{y$^{+t7.7}$ w^{+mC}=wHy}pum^{DG05207}/TM3, Sb^{1} Ser^{1}
21042	y^{1} w^{67c23}; P{y$^{+t7.7}$ w^{+mC}=wHy}pum^{DG05207}/TM3, Sb^{1} Ser^{1}
21214	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}koko^{EY16502}
22559	y^{1} w^{67c23}; P{w^{+mC} y$^{+mDint2}$=EPgy2}SelR^{EY22443}
22750	y^{1} w^{67c23}; Mi{ET1}cpx^{MB00267}
23491	w^{1118}; Mi{ET1}hug^{MB02782}
23750	y^{1} w^{67c23}; Mi{ET1}CG31140^{MB01635}/TM3, Sb^{1} Ser^{1}
24365	w^{1118}; Df(2L)BSC341/CyO
24635	P{w^{+mC}=VGlut-GAL4.D}1, w*
24635	P{VGlut-GAL4.D}1, w*
24976	w^{1118}; Df(3R)BSC472/TM6C, Sb^{1} cu^{1}
24981	w^{1118}; Df(3R)BSC477/TM6C, Sb^{1} cu^{1}
24994	w^{1118}; Df(3R)BSC490/TM6C, Sb^{1} cu^{1}
25006	w^{1118}; Df(3R)BSC502/TM6C, Sb^{1} cu^{1}
25010	w^{1118}; Df(3R)BSC506/TM6C, Sb^{1} cu^{1}
25013	w^{1118}; Df(3R)BSC509/TM6C, Sb^{1} cu^{1}

25057	w^{1118}; Df(3R)BSC529/TM6C, Sb^{1}
25126	w^{1118}; Df(3R)BSC568/TM6C, Sb^{1}
25275	w^{1118}; Mi{ET1}CG7675^{MB07646}/TM6C, Sb^{1}
25374	y^{1} w^{*}; P{Act5C-GAL4-w}E1/CyO
25374	y^{1} w^{*}; P{Act5C-GAL4-w}E1/CyO
25681	y^{1} w^{*}; P{w^{+mC}=npf-GAL4.1}2
25740	w^{1118}; Df(3R)BSC650/TM6C, Sb^{1} cu^{1}
25944	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01964}attP2
26518	w^{1118}; Df(3R)BSC666/TM6C, Sb^{1} cu^{1}
26518	w^{1118}; Df(3R)BSC666/TM6C, Sb^{1} cu^{1}
26534	w^{1118}; Df(3R)BSC682/TM6C, Sb^{1} cu^{1}
26580	w^{1118}; Df(3R)BSC728, P+PBac{w^{+mC}=XP3.RB5}BSC728/TM6C, Sb^{1} cu^{1}
26580	w^{1118}; Df(3R)BSC728, P+PBac{w^{+mC}=XP3.RB5}BSC728/TM6C, Sb^{1} cu^{1}
26724	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF02266}attP2
26838	w^{1118}; Df(3R)BSC740, P+PBac{w^{+mC}=XP3.RB5}BSC740/TM6C, Sb^{1} cu^{1}
27158	y^{1} w^{*}; P{w^{+mC}=EP}Rop^{G4478}/TM3, Sb^{1} Ser^{1}
27237	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF02555}attP2
27363	w^{1118}; Df(3R)BSC791/TM6C, Sb^{1} cu^{1}
27536	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF02687}attP2/TM3, Sb^{1}
27722	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF02804}attP2
27920	w^{1118}; Df(3R)BSC847/TM6C, Sb^{1} cu^{1}
28515	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HM05001}attP2
28617	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03032}attP2
28648	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03063}attP2
28697	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03113}attP2
28705	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03122}attP2
28723	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03150}attP2/TM3, Sb^{1}
28786	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03214}attP2
28929	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HM05140}attP2
28933	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HM05144}attP2/TM3, Sb^{1}
29322	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF02483}attP2/TM3, Sb^{1}
30026	y^{1} w^{1118}; P{w^{+m*}=GawB}GH146
30620	y^{1} w^{*}; Mi{$y^{+mDint2}$=MIC}CG31030^{MI00107}/TM3, Sb^{1} Ser^{1}
30819	w^{*}; P{$w^{+mW.hs}$=GawB}Kdm2^{36Y}
31311	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01258}attP2/TM3, Ser^{1}
31322	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01273}attP2
31334	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01291}attP2/TM3, Ser^{1}
31348	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01306}attP2
31471	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01245}attP2/TM3, Sb^{1}
31484	y^{1} v^{1}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF01324}attP2
31803	w^{1118}; P{w^{+mC}=EP}CG5916^{G13766}
31839	w^{1118}; P{w^{+mC}=EP}CG14309^{G19121}

32040	P{w^{+m*}=Appl-GAL4.G1a}1, y^{1} w*
32957	y^{1} sc* v^{1}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMS00751}attP2
32986	y^{1} sc* v^{1}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMS00786}attP2
33615	y^{1} v^{1}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMS00007}attP2
33894	y^{1} sc* v^{1}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMS00832}attP2
34391	y^{1} sc* v^{1}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMS01385}attP2
34969	y^{1} sc* v^{1}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMS00884}attP2
36065	y^{1} sc* v^{1}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.GL00423}attP2
36336	w*; CyO, P{w^{+mC}=2xTb^{1}-RFP}CyO/T(2;3)ap^{Xa}
36337	FM7c, P{w^{+mC}=2xTb^{1}-RFP}FM7c, sn^{+}/oc$^{otd\text{-}XC86}$
45052	w^{1118}; P{w^{+mC}=GMR14A10-GAL4}attP2
45054	w^{1118}; P{w^{+mC}=GMR14B10-GAL4}attP2
45224	w^{1118}; P{w^{+mC}=GMR34F05-GAL4}attP2
45676	w^{1118}; P{w^{+mC}=GMR39E11-GAL4}attP2/TM3, Sb^{1}
46021	w^{1118}; P{w^{+mC}=GMR51F02-GAL4}attP2
46027	w^{1118}; P{w^{+mC}=GMR52D08-GAL4}attP2
46058	w^{1118}; P{w^{+mC}=GMR54F12-GAL4}attP2
46066	w^{1118}; P{w^{+mC}=GMR55B11-GAL4}attP2
46067	w^{1118}; P{w^{+mC}=GMR55C06-GAL4}attP2
46075	w^{1118}; P{w^{+mC}=GMR55G09-GAL4}attP2
46373	w^{1118}; P{w^{+mC}=GMR57D03-GAL4}attP2
46539	w^{1118}; P{w^{+mC}=GMR64D08-GAL4}attP2
46635	w^{1118}; P{w^{+mC}=GMR70F02-GAL4}attP2
46649	w^{1118}; P{w^{+mC}=GMR71A08-GAL4}attP2
46664	w^{1118}; P{w^{+mC}=GMR71G07-GAL4}attP2
46899	w^{1118}; P{w^{+mC}=GMR89H08-GAL4}attP2/TM3, Sb^{1}
46962	w^{1118}; P{w^{+mC}=GMR76B09-GAL4}attP2
47019	w^{1118}; P{w^{+mC}=GMR78E03-GAL4}attP2
47245	w^{1118}; P{w^{+mC}=GMR94A06-GAL4}attP2
47269	w^{1118}; P{w^{+mC}=GMR95A01-GAL4}attP2
47634	w^{1118}; P{w^{+mC}=GMR52A01-GAL4}attP2

Table B.2

Overview of Exelixis deficiency screen in a *sda* background

Exelixis Number	Stock Number	% paralyzed	Fold change	# tested
Df(3R)Exel6140	7619	5.4	0.9	74
Df(3R)Exel6141	7620	0.0	0.0	60
Df(3R)Exel6142	7621	0.0	0.0	60
Df(3R)Exel6143	7622	0.0	0.0	68
Df(3R)Exel6144	7623	13.3	2.2	60
Df(3R)Exel6145	7624	11.7	1.9	60
Df(3R)Exel6146	7625	8.0	1.3	50
Df(3R)Exel6147	7626	7.7	1.2	65
Df(3R)Exel6148	7627	0.0	0.0	95
Df(3R)Exel6149	7628	0.0	0.0	67
Df(3R)Exel6150	7629	23.1	3.7	52
Df(3R)Exel6151	7630	63.3	10.2	120
Df(3R)Exel6152	7631	0.0	0.0	60
Df(3R)Exel6153	7632	0.0	0.0	82
Df(3R)Exel6154	7633	50.8	8.2	59
Df(3R)Exel6155	7634	10.0	1.6	60
Df(3R)Exel6156	7635	1.7	0.3	60
Df(3R)Exel6157	7636	11.7	1.9	60
Df(3R)Exel6158	7637	12.0	1.9	75
Df(3R)Exel6159	7638	15.8	2.5	76
Df(3R)Exel6160	7639	11.7	1.9	60
Df(3R)Exel6161	7640	23.9	3.9	71
Df(3R)Exel6162	7641	1.6	0.3	64
Df(3R)Exel6163	7642	5.2	0.8	58
Df(3R)Exel6164	7643	8.3	1.3	60
Df(3R)Exel6165	7644	60.0	9.7	60
Df(3R)Exel6166	7645	3.3	0.5	60
Df(3R)Exel6167	7646	20.0	3.2	60
Df(3R)Exel6168	7647	0.0	0.0	72
Df(3R)Exel6169	7648	0.0	0.0	60
Df(3R)Exel6170	7649	1.7	0.3	60
Df(3R)Exel6171	7650	0.0	0.0	75
Df(3R)Exel6172	7651	20.0	3.2	60
Df(3R)Exel6173	7652	3.2	0.5	62
Df(3R)Exel6174	7653	1.4	0.2	69
Df(3R)Exel6176	7655	0.0	0.0	67
Df(3R)Exel6178	7657	21.7	3.5	60

Exelixis Number	Stock Number	% paralyzed	Fold change	# tested
Df(3R)Exel6179	7658	2.3	0.4	88
Df(3R)Exel6180	7659	0.0	0.0	66
Df(3R)Exel6181	7660	20.0	3.2	60
Df(3R)Exel6182	7661	0.0	0.0	57
Df(3R)Exel6184	7663	0.0	0.0	60
Df(3R)Exel6185	7664	0.0	0.0	60
Df(3R)Exel6186	7665	0.0	0.0	60
Df(3R)Exel6187	7666	1.0	0.2	98
Df(3R)Exel6188	7667	0.0	0.0	60
Df(3R)Exel6189	7668	14.9	2.4	67
Df(3R)Exel6190	7669	5.5	0.9	55
Df(3R)Exel6191	7670	9.1	1.5	77
Df(3R)Exel6192	7671	0.0	0.0	60
Df(3R)Exel6193	7672	6.7	1.1	60
Df(3R)Exel6194	7673	1.7	0.3	60
Df(3R)Exel6195	7674	0.0	0.0	69
Df(3R)Exel6196	7675	43.3	7.0	60
Df(3R)Exel6197	7676	3.6	0.6	56
Df(3R)Exel6198	7677	0.0	0.0	60
Df(3R)Exel6199	7678	0.0	0.0	60
Df(3R)Exel6200	7679	0.0	0.0	60
Df(3R)Exel6201	7680	0.0	0.0	54
Df(3R)Exel6202	7681	5.0	0.8	60
Df(3R)Exel6203	7682	18.3	3.0	60
Df(3R)Exel6204	7683	8.3	1.3	60
Df(3R)Exel6205	7684	33.3	5.4	51
Df(3R)Exel6206	7685	9.4	1.5	64
Df(3R)Exel6208	7686	15.0	2.4	60
Df(3R)Exel6209	7687	0.0	0.0	60
Df(3R)Exel6210	7688	0.0	0.0	65
Df(3R)Exel6211	7689	3.3	0.5	60
Df(3R)Exel6212	7690	5.1	0.8	59
Df(3R)Exel6213	7691	0.0	0.0	92
Df(3R)Exel6214	7692	0.0	0.0	19
Df(3R)Exel6215	7693	0.0	0.0	60
Df(3R)Exel6216	7694	0.0	0.0	82
Df(3R)Exel6217	7695	11.7	1.9	60
Df(3R)Exel6218	7696	1.5	0.2	65
Df(3R)Exel6259	7726	0.0	0.0	65
Df(3R)Exel6263	7730	0.0	0.0	1
Df(3R)Exel6264	7731	0.0	0.0	60

Exelixis Number	Stock Number	% paralyzed	Fold change	# tested
Df(3R)Exel6265	7732	11.7	1.9	60
Df(3R)Exel6267	7734	3.3	0.5	60
Df(3R)Exel6269	7736	0.0	0.0	60
Df(3R)Exel6270	7737	71.7	11.6	60
Df(3R)Exel6273	7740	0.0	0.0	53
Df(3R)Exel6274	7741	2.9	0.5	69
Df(3R)Exel6275	7742	3.3	0.5	60
Df(3R)Exel6276	7743	3.4	0.5	59
Df(3R)Exel6280	7746	0.0	0.0	60
Df(3R)Exel6288	7752	0.0	0.0	60
Df(3R)Exel9020	7917	3.4	0.5	59
Df(3R)Exel8194	7918	18.3	3.0	60
Df(3R)Exel7379	7919	36.5	5.9	63
Df(3R)Exel7315	7931	15.5	2.5	58
Df(3R)Exel7317	7932	40.4	6.5	52
Df(3R)Exel7357	7948	0.0	0.0	60
Df(3R)Exel9029	7951	0.0	0.0	60
Df(3R)Exel7283	7952	0.0	0.0	78
Df(3R)Exel7284	7953	3.3	0.5	60
Df(3R)Exel8143	7954	1.7	0.3	60
Df(3R)Exel9036	7955	16.7	2.7	60
Df(3R)Exel7305	7956	35.0	5.6	60
Df(3R)Exel7306	7957	1.7	0.3	60
Df(3R)Exel8152	7958	5.0	0.8	60
Df(3R)Exel7308	7959	0.0	0.0	60
Df(3R)Exel7309	7960	0.0	0.0	60
Df(3R)Exel8154	7961	13.3	2.2	60
Df(3R)Exel9018	7962	21.7	3.5	60
Df(3R)Exel8153	7963	5.0	0.8	60
Df(3R)Exel9019	7964	6.7	1.1	60
Df(3R)Exel7310	7965	1.7	0.3	60
Df(3R)Exel7312	7966	33.3	5.4	60
Df(3R)Exel8155	7967	0.0	0.0	60
Df(3R)Exel7313	7968	10.0	1.6	80
Df(3R)Exel7314	7969	0.0	0.0	53
Df(3R)Exel7316	7970	5.4	0.9	74
Df(3R)Exel7318	7972	5.0	0.8	60
Df(3R)Exel8157	7973	32.4	5.2	71
Df(3R)Exel8158	7974	11.7	1.9	60
Df(3R)Exel7320	7975	13.2	2.1	53
Df(3R)Exel8159	7976	0.0	0.0	52

Exelixis Number	Stock Number	% paralyzed	Fold change	# tested
Df(3R)Exel7321	7977	16.7	2.7	60
Df(3R)Exel7326	7980	0.0	0.0	60
Df(3R)Exel8162	7981	2.8	0.4	72
Df(3R)Exel7327	7982	5.7	0.9	70
Df(3R)Exel7329	7984	0.0	0.0	57
Df(3R)Exel7330	7985	3.3	0.5	60
Df(3R)Exel8163	7987	1.6	0.3	64
Df(3R)Exel8165	7988	0.0	0.0	60
Df(3R)Exel9030	7989	28.3	4.6	60
Df(3R)Exel9012	7990	0.0	0.0	60
Df(3R)Exel9013	7991	23.3	3.8	60
Df(3R)Exel9014	7992	10.0	1.6	70
Df(3R)Exel8178	7993	3.3	0.5	60
Df(3R)Exel9056	7994	3.0	0.5	99
Df(3R)Exel9025	7995	23.3	3.8	60
Df(3R)Exel7378	7997	1.5	0.2	67

Table B.3
Bang-sensitivity in *para*^{bss1} crossed to seizure-enhancing Exelixis deficiencies

Exelixis Deficiency	Stock Number	% Paralyzed	Fold change	# tested
Df(3R)Exel6150	7629	40.0	1.0	60
Df(3R)Exel6151	7630	95.9	2.5	73
Df(3R)Exel6154	7633	69.8	1.8	53
Df(3R)Exel6161	7640	68.3	1.8	60
Df(3R)Exel6165	7644	38.7	1.0	75
Df(3R)Exel6178	7657	71.2	1.8	73
Df(3R)Exel6196	7675	45.2	1.2	73
Df(3R)Exel6270	7737	76.7	2.0	60
Df(3R)Exel7305	7956	95.0	2.4	60
Df(3R)Exel7317	7932	96.2	2.5	52
Df(3R)Exel7328	7983	98.5	2.5	68
Df(3R)Exel7379	7919	85.0	2.2	60
Df(3R)Exel8157	7973	100.0	2.6	58
Df(3R)Exel9013	7991	44.9	1.1	49
Df(3R)Exel9025	7995	81.1	2.1	53
control				
w1118	5905	39.0	1.0	105

Table B.4
Bang-sensitivity in *eas* crossed to seizure-enhancing Exelixis deficiencies

Exelixis Deficiency	Stock Number	% paralyzed	Total N
Df(3R)Exel6150	7629	0	58
Df(3R)Exel6151	7630	11.5	52
Df(3R)Exel6154	7633	0.0	80
Df(3R)Exel6161	7640	0.0	87
Df(3R)Exel6165	7644	0.0	67
Df(3R)Exel6178	7657	0.0	76
Df(3R)Exel6196	7675	0.0	53
Df(3R)Exel6270	7737	2.2	89
Df(3R)Exel7305	7956	0.0	64
Df(3R)Exel7317	7932	0.0	63
Df(3R)Exel7328	7983	0.0	61
Df(3R)Exel7379	7919	0.0	59
Df(3R)Exel8157	7973	0.0	62
Df(3R)Exel9013	7991	0.0	51
Df(3R)Exel9025	7995	0.0	53
control			
w1118	5905	0	71

Table B.5
Bang-sensitivity testing of *sda* crossed to deficiencies overlapping seizure-enhancing Exelixis deficiencies

Stock Number	Genotype	% paralyzed	Fold change	# of flies tested
7661	Df(3R)Exel6182	0.0	0.0	60
7731	Df(3R)Exel6264	2.5	0.2	40
7969	Df(3R)Exel7314	10.4	0.9	48
8968	Df(3R)ED5516	2.5	0.2	40
9086	Df(3R)ED5591	16.7	1.5	60
9206	Df(3R)ED5573	16.7	1.5	24
9225	Df(3R)ED5301	5.0	0.4	60
9227	Df(3R)ED5428	20.5	1.8	44
9338	Df(3R)ED5296	7.7	0.7	26
9482	Df(3R)ED10642	33.3	3.0	60
9699	Df(3R)BSC222	27.4	2.4	62
24976	Df(3R)BSC472	0.0	0.0	60
24981	Df(3R)BSC477	13.9	1.2	36
24994	Df(3R)BSC490	13.8	1.2	29
25006	Df(3R)BSC502	8.9	0.8	45
25013	Df(3R)BSC509	41.7	3.7	60
25057	Df(3R)BSC529	0.0	0.0	60
25126	Df(3R)BSC568	36.7	3.3	60
25740	Df(3R)BSC650	0.0	0.0	86
26534	Df(3R)BSC682	6.7	0.6	60
26580	Df(3R)BSC728	3.3	0.3	60
26838	Df(3R)BSC740	9.4	0.8	53
27363	Df(3R)BSC791	20.0	1.8	60
27920	Df(3R)BSC847	1.7	0.1	60

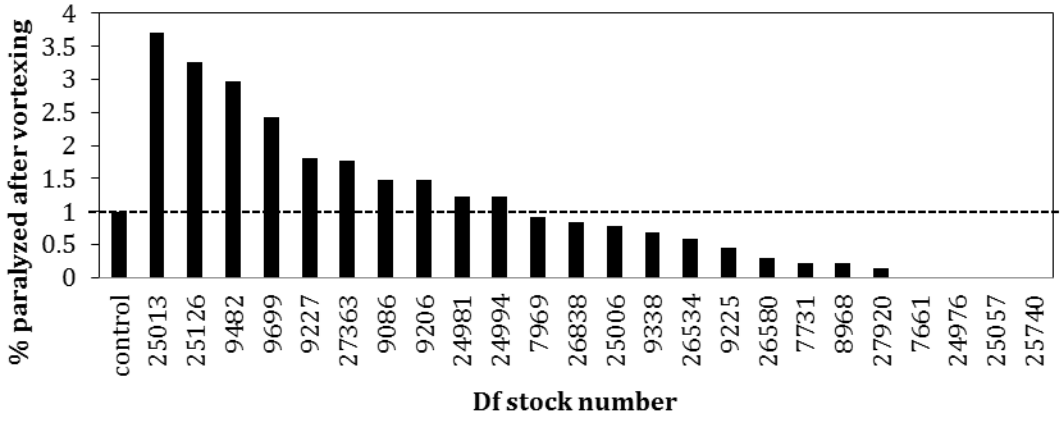


Figure B.1 Bang-sensitivity testing of *sda* crossed to deficiencies overlapping seizure-enhancing Exelixis deficiencies. These deficiencies overlap with the Exelixis deficiencies found to be enhancers in the screen. Control is *sda*/+. N≥24 for each deficiency.

Table B.6
Bang-sensitivity of transposon insertion alleles crossed to *slamdance*

Gene	Associated Exel Df	% Paralyzed	Fold Difference	Number Tested
w1118	5905	6.1	1	180
Akt	7983	35.0	5.7	20
CG10324	7737	0.0	0.0	34
CG11140	7675	63.8	10.4	80
CG14309	7657	16.3	2.7	80
CG18005	7630	15.0	2.5	60
CG31140	7675	18.3	3.0	60
CG5903	7983	0.0	0.0	20
CG5916	7983	3.8	0.6	80
CG6574	7956	1.7	0.3	60
CtBP	7973	11.3	1.8	80
Fas1	7737	0.0	0.0	60
Glut4ef	7633	17.5	2.9	80
GycBeta	7919	36.7	6.0	60
Hug	7932	11.7	1.9	60
kdm2	7630	13.3	2.2	60
Koko	7657	10.0	1.6	80
Mcm5	7638	11.7	1.9	60
nonA-1	7737	11.4	1.9	70
Pros	7962	0.0	0.0	60
Pum	7630	8.8	1.4	80
Repo	7657	1.0	0.2	100

Table B.7
Bang-sensitivity of RNAi lines crossed to *elav-GAL4*; *sda*

Target Gene	Associated Exel Stock	% paralyzed	Fold difference	# tested
control		2.5	1	80
AdoR	7692	12.5	5	80
Art1	7956	7.0	2.8	100
ATPsyngamma	7995	1.4	0.6	69
ccty	7737	0.0	0	17
CG18549	7932	0.0	0	100
CG18616	7932	7.7	3.1	13
CG3747	7730	5.0	2	100
CG42342	7737	15.0	6	80
CG4596	7956	6.3	2.5	80
CG9467	7633	10.0	4	80
CtBP	7973	0.0	0	80
fbx011	7633	1.3	0.5	80
fer2	7983	16.3	6.5	80
GycBeta	7919	13.8	5.5	80
hug	7932	4.0	1.6	100
npf	7737	5.0	2	100
sima	7692	31.3	12.5	80
Tsp86D	7956	10.0	4	80
yata	7995	7.5	3	80

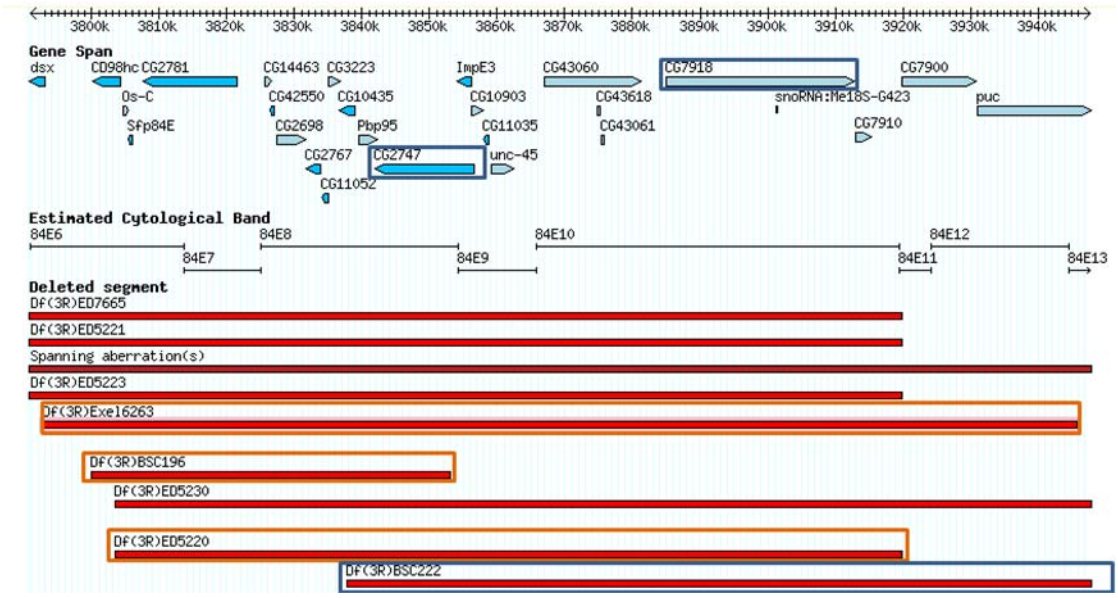


Figure B.2 Map of deletions and alleles within Exel6263 tested for synthetic lethality with *sda*. Orange boxes represent genotypes that resulted in synthetic lethality. Blue boxes represent viable crosses.

Table B.8
Candidate genes within Exel6263 that may cause partial synthetic lethality with *sda*

Candidate Genes	Function	Expression
CD98hc	cation binding, leucine import	high in most tissues
CG10435	unknown	high in most tissues
CG11052	acylphosphatase activity	high in testis
CG14463	phosphatidylinositol N-acetylglucosaminyltransferase activity	low in most tissues
CG2698	unknown	moderate in nervous system and other tissues
CG2767	alcohol dehydrogenase	low in nervous system, high in eye/crop
CG2781	fatty acid synthesis	high in gut and head
CG3223	unknown	moderate in most tissues
CG42550	unknown	unknown
Os-C	pheremone binding	high in head
Pbp95	DNA binding, lateral inhibition	high in ovaries, low in most tissues
Sfp84E	Reproduction	unknown

Table B.9
Genes within Exel6214 tested for lethality in combination with *sda*

Stock Description	Function	Viable with *sda*?
Exel6214		semilethal
Adenosine receptor (*AdoR*)	Synaptic transmission	viable
similar (*sima*)	Transcription regulation	viable
CG31030	ATP hydrolysis	viable

Table B.10
Summary of all deficiencies and alleles tested by crossing to *sda*

Exelixis Deficiency	Gene	Type	Stock Number	Fold Change
Df(3R)Exel6151			7630	10.4
	Df(3R)ED5296	Deficiency	9338	1.3
	Kdm2	P-element	30819	2.2
	CG18995	P-element	15965	2.5
	pum	P-element	21042	3.3
	pum	P-element	11544	3.7
Df(3R)Exel6154			7633	8.3
	Teh1	P-element	13221	0.0
	Glut4ef	P-element	16364	2.9
	CG9467	RNAi	26724	4.0
	fbx011	RNAi	31484	0.5
Df(3R)Exel6178			7657	3.6
	Df(3R)BSC650	Deficiency	25740	0.0
	Df(3R)BSC682	Deficiency	26534	1.1
	Df(3R)BSC509	Deficiency	25013	6.8
	repo	P-element	11604	0.2
	koko	P-element	21214	1.6
	CG14309	P-element	31839	2.7
Df(3R)Exel6196			7675	7.1
	Df(3R)Exel9014	Deficiency	7992	1.6
	CG31140	P-element	23750	3.0
	CG11140	P-element	13427	10.5
Df(3R)Exel6270			7737	11.7
	Df(3R)BSC472	Deficiency	24976	0.0
	Df(3R)ED10642	Deficiency	9482	5.5
	Fas1	P-element	16029	0.0
	npf RNAi	RNAi	27237	0.8
	nonA-1	P-element	16690	1.9
	CG10324	P-element	19012	0.0
	CG42342	RNAi	28628	6.0
	ccty	P-element	34969	0.0
Df(3R)Exel7379			7919	6.0
	GycB	P-element	15221	6.0
	GycB	RNAi	28786	5.5
Df(3R)Exel7317			7932	6.6
	Df(3R)BSC847	Deficiency	27920	0.3
	Df(3R)ED5573	Deficiency	9206	2.7
	hug	RNAi	28705	1.6
	hug	P-element	23491	1.9
	CG18616	RNAi	32957	3.1
	CG18549	RNAi	34391	0.0
Df(3R)Exel7305			7956	5.7

	Df(3R)BSC529	Deficiency	25057	0.0
	Df(3R)BSC568	Deficiency	25126	6.0
	Art1	RNAi	31348	1.1
	CG6574	P-element	17837	0.3
	Tsp86D	RNAi	28515	4.0
	CG4596	RNAi	28617	2.5
Df(3R)Exel7328			7983	3.8
	Df(3R)Exel7327	Deficiency	7982	0.9
	Df(3R)BSC728	Deficiency	26580	0.3
	Akt	P-element	11627	5.7
	CG5903	P-element	20714	0.0
	CG5916	P-element	31803	0.6
	fer2	RNAi	28697	6.5
Df(3R)Exel9025			7995	3.8
	ATPsyngamma	RNAi	28723	0.6
	yata	RNAi	32986	3.0

Table B.11
Exelixis deficiency enhancers of *sda* with no identified enhancer genes

Exelixis Deficiency	Gene	Type	Stock Number	Fold Change
Df(3R)Exel6150			7629	3.8
	Df(3R)BSC477	Deficiency	24981	2.3
Df(3R)Exel6161			7640	3.9
		Deficiency	8968	0.4
		Deficiency	7743	0.6
Df(3R)Exel6165			7644	9.8
	Df(3R)BSC740	Deficiency	26838	1.5
	Df(3R)ED5591	Deficiency	9086	2.7
Df(3R)Exel8157			7973	5.3
	CtBP	Transposon	11590	1.6
	CtBP	RNAi	31334	0.0
Df(3R)Exel9013			7991	3.8
	Df(3R)BSC490	Deficiency	24994	2.3
	WASp	Transposon	25955	0.0

APPENDIX C

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

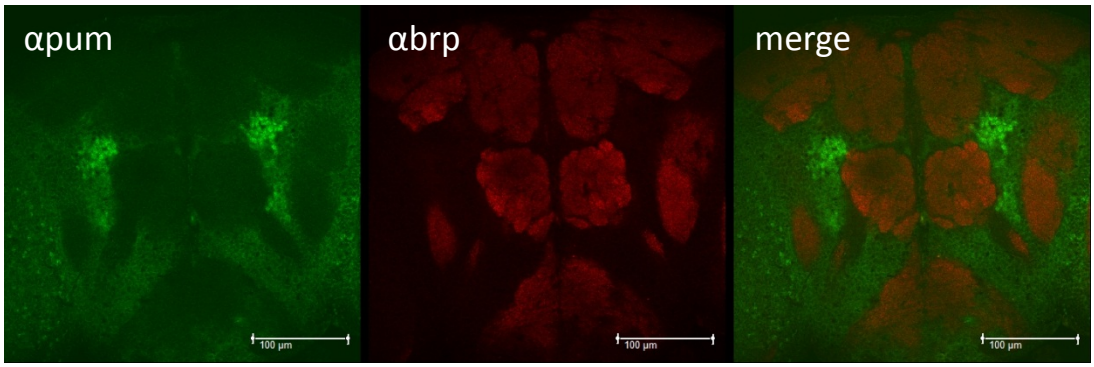


Figure C.1 Double staining of pum and synaptic marker brp in adult brains. Confocal images were taken of the central brain region. Expression of pum is separate from the neuropil labeled by brp. Scale bars are 100 µm.

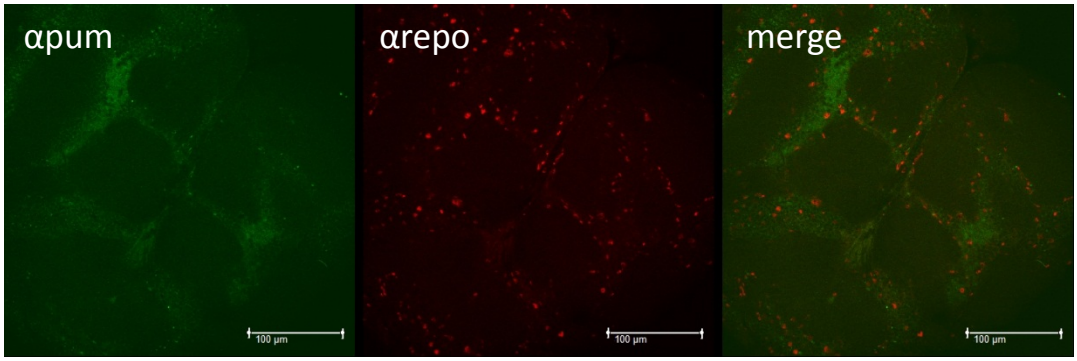
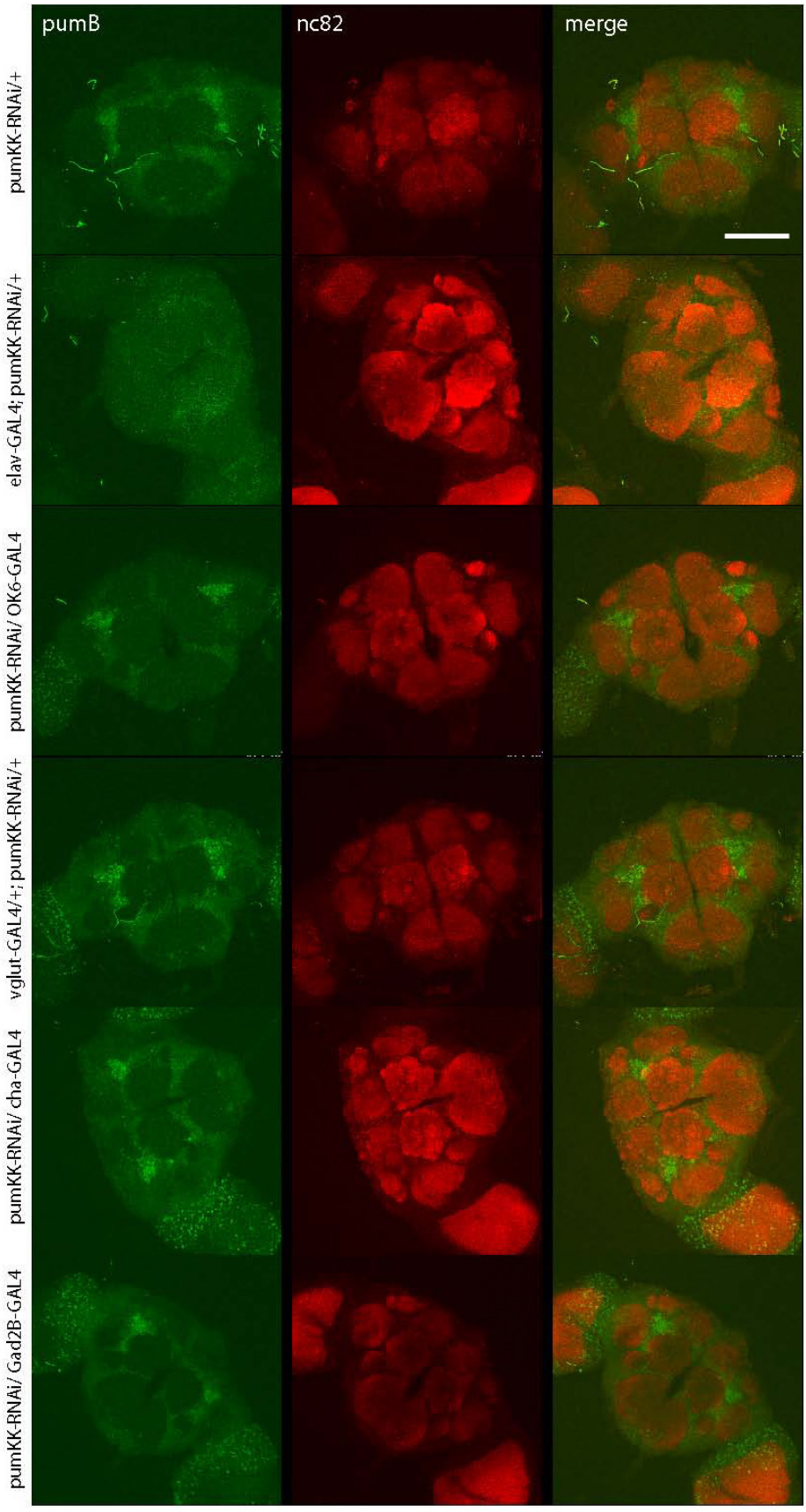


Figure C.2 Double staining of pum and repo, a glial marker, in adult brains. Confocal image were taken of the central brain region. Cells positive for repo do not overlap those labeled for pum. Scale bars, 100 µm.

Figure C.3 Double staining of pum and nc82 (brp), a synaptic marker, after targeted knockdown of pum by several GAL4 lines including elav (panneuronal), OK6 (motor neurons), VGlut (glutamatergic neurons), Cha (cholinergic neurons), and Gad2B (GABAergic neurons). Scale bar, 100 μm.


pumB
nc82
merge
pumKK-RNAi/+
elav-GAL4; pumKK-RNAi/+
pumKK-RNAi/ OK6-GAL4
vglut-GAL4/+; pumKK-RNAi/+
pumKK-RNAi/ cha-GAL4
pumKK-RNAi/ Gad2B-GAL4

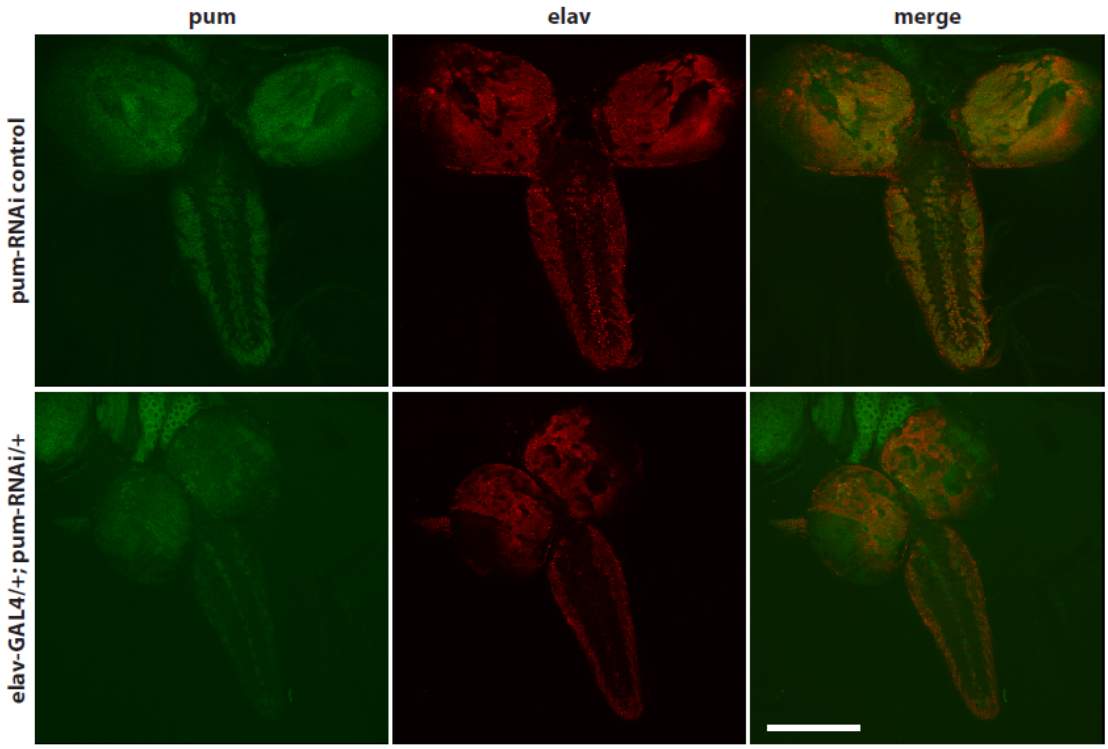


Figure C.4 Knockdown of pumilio using RNAi targeted to all neurons (elav) in the larval brain. Confocal images of third instar larvae brains stained with pumilio and elav. Scale bar, 50 μm.

Table C.1
Behavior testing after knockdown of pumilio in various cell types by RNAi

Genotype	Targeted Cell Type	# paralyzed	N	% seized	Fold change
$para^{bss1}$/+ ; pum-RNAi/+	control	72	155	46.5	1.0
$para^{bss1}$/elav-GAL4; pum-RNAi/+	panneuronal	50	50	100.0	2.2
$para^{bss1}$/appl –GAL4; pum-RNAi/+	panneuronal	88	96	91.7	2.0
$para^{bss1}$/+ ; pum-RNAi/tdc2	octopaminergic neurons	16	21	76.2	1.6
$para^{bss1}$/vglut –GAL4; pum-RNAi/+	glutamatergic neurons	14	28	50.0	1.1
$para^{bss1}$/+ ; VaCHT-GAL4/pum-RNAi	cholinergic neurons	20	21	95.2	2.1
$para^{bss1}$/+ ; cha-GAL4/pum-RNAi	cholinergic neurons	65	71	91.5	2.0
$para^{bss1}$/+ ; slit-GAL4/+; pum-RNAi/+	glia	11	33	33.3	0.7
$para^{bss1}$/+ ; repo-GAL4/+; pum-RNAi/+	glia	22	40	55.0	1.2
$para^{bss1}$/+ ; gad1-GAL4/pum-RNAi	GABA-ergic neurons	23	37	62.2	1.3

Table C.2

Behavior testing after knockdown of Pum by RNAi in a *slamdance* background

Genotype	Target	N	# Paralyzed	% Paralyzed	Fold Change
pum-RNAi/+; sda/+	control	80	4	5.0	1.0
elav-GAL4, pum-RNAi/+; sda/+	panneural	80	65	81.3	16.3
Gad2B/pum-RNAi; sda/+	GABAergic neurons	80	63	78.8	15.8
pum-RNAi/VAChT-GAL4; sda/+	cholinergic neurons	119	49	41.2	8.2
pum-RNAi/Tdc2-GAL4; sda/+	octopaminergic neurons	80	23	28.8	5.8
Vglut-GAL4/+; pum-RNAi/+; sda/+	glutamatergic neurons	80	17	21.3	4.3
pum-RNAi/+; sda/slit-GAL4	glia	80	10	12.5	2.5
pum-RNAi/cha-GAL4; sda/+	cholinergic neurons	65	7	10.8	2.2

Table C.3
Seizure threshold in flies with neuron-targeted pum knockdown

Genotype	Seizure threshold ± SE (V)	Number tested
para^{bss1}/+ ; *pum*-RNAi/+	10.58 ± 1.22	12
para^{bss1}, *elav-GAL4*/+ ; *pum*-RNAi/+	7.56 ± 0.53	9

Table C.4
Effects of pumilio overexpression on larval survival

Age of larvae at time of heat shock (days)	Life cycle stage	% survival with heat shock	% survival without heat shock
1	First Instar	100.0	100.0
2		96.9	97.4
3	Second Instar	100.0	100.0
4		100.0	100.0
5	Third Instar	100.0	100.0
6		97.4	98.2
7	Wandering Third Instar	77.4	100.0
8	Pupa	64.7	100.0
10		95.8	100.0